Full Length Research Paper

Evaluation of lima bean flour fermented with *Lactobacillus* sp. as a probiotic food

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The probiotic potential of lima bean flour fermented with *Lactobacillus* sp. *in vitro* and *in vivo* was studied. 3 species of *Lactobacillus* including *Lactobacillus fermentum*, *Lactobacillus plantarum* and *Lactobacillus cellebiosus* were isolated from traditionally prepared 'ogi' made from white and yellow maize (*Zea mays*) and from red guinea corn (*Sorghum bicolor*), after which they were screened for their growth and survival in Lima bean flour. Among all the species, *L. plantarum* showed appreciable growth (9.3×10^6 cfu/ml) after 72 h fermentation at 37°C. On storing the fermented products for 14 days at 24 ± 2°C, no marked change in the viable count of this species was observed. In contrast, the number of other species, that is, *L. fermentum* and *L. cellebiosus* reduced. There was marked increase in bacterial counts of all the products after storage at room temperature (24 ± 2°C) for 14 days compared to those stored at refrigeration temperature (4 ± 2°C). The physicochemical analysis of the fermented samples showed increase in total titratable acidity (TTA) and temperature with a gradual reduction in pH. There was an increase in protein content and decrease in carbohydrate, ash and moisture contents in the fermented sample compared with the unfermented sample. Under different pH ranges, *L. plantarum* also showed appreciable growth and survival at pH 2 to 3. Supplementing the diet of albino rats infected with *Escherichia coli* with the fermented product increased significantly the number of *Lactobacilli*, compared to the control and at the same time, the number of *E. coli* and other feacal enterobacteria decreased significantly. This study revealed that Lima bean flour fermented with *L. plantarum* could be used as an excellent probiotic food.

Key words: *Lactobacillus* species, lima beans, fermentation, probiotics and gastrointestinal tolerance.

INTRODUCTION

The term "probiotics" was first introduced by Mietchnikoff (1908). Probiotics have been defined as living bacteria and supportive substances that have beneficial effects on the host by improving the bacterial balance in the intestine (Fuller, 1989). Probiotics are live microbial feed or food supplements that beneficially affect the host by improving its intestinal microbial balance. In addition, the following properties and functions have been attributed to probiotics: they adhere to host epithelial tissue; they are acid resistant and bile tolerant; they are safe, non-pathogenic and non-carcinogenic; they cause improve-ment of the intestinal microflora; they have a cholesterol lowering, immuno-stimulating and allergy lowering effect;

they are also known to synthesize and enhance the bioavailability of nutrients (Sanders, 2003). Probiotics are commonly consumed as part of fermented foods with specially added active live cultures; such as in yogurt, soy yogurt, or as dietary supplements (Guarner and Scaafsma, 1998). Among a number of functional properties attributed to foods, probiotics take a centre stage. Vasiljevic and Shah (2008) reported that the World Health Organization (WHO) in 1994 deemed probiotics to be the next most important immune defense systems as a result of increasing antibiotic resistance of commonly prescribed antibiotics. Lima beans are a type of legume native to South America. It is named after the capital city of Peru, Lima and has been grown there since 6000 BC. This highly nutritious beans is grown for its seed, which is eaten as a vegetable. It contains both soluble fiber, which helps regulate blood sugar levels and lowers cholesterol,

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and insoluble fiber, which prevents constipation, digestive disorders, irritable bowel syndrome and diverticulitis (Wootten, 1995). The objective of this study was to evaluate the probiotic value of lima bean flour fermented with *Lactobacillus* sp.

MATERIALS AND METHODS

Source of organisms

Lactobacillus sp. (*L. plantarum, L. cellebiosus* and *L. fermentum*) used in this study were isolated from traditionally prepared 'ogi' made from *Zea mays* and *Sorghum bicolor*.

Enterotoxigenic strain of *E. coli* (0157:H7) was collected from the Department of Microbiology, Federal University of Technology, Akure, Ondo State, Nigeria.

Source of lima bean grains

Lima bean grains were purchased from Koko market in Owo, Ondo state. Prior to further use, the grains were sorted, cleaned, boiled (to get rid of the toxins), dried and ground into fine flour to pass a 0.4 mm screen.

Source of experimental animals

25 albino rats (*Rattus norvegicus*) (6 to 8 weeks old) were purchased from the Department of Animal Production and Health, Federal University of Technology, Akure, Nigeria. They were housed in stainless steel cages under controlled conditions and placed on a basal diet purchased from Top feeds, Sapele, Delta State, Nigeria.

Reagents /chemicals

All reagents and chemicals were obtained from the Department of Microbiology, Federal University of Technology, Akure, Ondo State Nigeria.

Experimental design

Culturing and harvesting of Lactobacillus cells

Overnight broth cultures of test isolates A, B, and C were centrifuged at 10,000 g for 15 min. The pellets were rinsed out thrice with 10 ml phosphate buffer saline (PBS) into sterilized universal bottles and kept as stock cultures in the refrigerator at 4 ± 2°C. The total viable cell in the stock solution was then determined using serial dilution and pour plate methods.

Fermentation and storage

Lima beans flour was mixed with distilled water (1:3) in 7 fermentation jars (A1, A2, B1, B2, C1, C2 and D) which were autoclaved at 121°C for 15 min. Jars were allowed to cool after which each jar was inoculated with 10^7 cfu/ml each of the test isolates *L. fermentum*, *L. plantarum*, and *L. cellebiosus* with A1 and A2 containing *L. fermentum*, B1 and B2 containing *L. plantarum*, C1 and C2 containing *L. cellebiosus* and D was uninoculated serving as the control. After thorough mixing, the properly corked jars were incubated anaerobically at 37°C for 72 h for fermentation to take place. After, fermentation jars A1, B1 and C1 were stored at 4 ± 2°C while A2, B2 and C2 were stored at 25 ± 2°C (room temperature) for 14 days respectively. Viable counts of LAB in the products were determined during the period of fermentation and after storage.

Microbial analysis

Samples collected during the fermentation (at 0, 24, 48 and 72 h) and storage (after 14 days at 4 and 24°C respectively) were used for bacterial enumeration using serial dilution and pour plate method on De Mann Rogosa and Sharpe Agar. Plates were incubated anaerobically at 37°C for 48 h to determine the best species in terms of growth and survival in Lima beans.

Physicochemical analyses

pH and total titrable acidity

A mixture of 10 g of each fermented product was used for pH determination as described in AOAC (1995). Total titratable acidity (TTA) was determined by titrating 20 ml of the same sample against 0.1 M NaOH.

Proximate composition

The moisture, crude fibre, fat, protein (N×6.25), ash and carbo-hydrate contents of both the fermented and unfermented samples were determined using relevant methods described previously (AOAC, 1995).

In vitro studies of gastrointestinal tolerance

Isolate's tolerance to different acidic conditions was tested by centrifuging overnight culture of the test isolate for 10 min at 3000 rpm. The pellet was then resuspended in the same volume of saline solution (9.8 g of NaCl in 1000 ml of distilled water). 1 ml of this dilution (pellet in saline solution) was plated for each of the isolates; this was done so as to estimate the number of viable cells that will be subjected to the acidic pH. 9 ml of sterile distilled water that had already been adjusted to pH 2, 3, 4 and 5, using phosphate buffer was transferred into already labeled test tubes, which was done in triplicate for each isolate. Then, 1 ml of the resuspended pellet containing the isolates were inoculated into the appropriate test tubes, this was agitated and incubated at 37°C for 3 h. After the three hours incubation, the appropriate dilutions was plated on De Mann Rogosa and Sharpe Agar and incubated anaerobically at 37°C for 48 h. After subjecting the different isolates to different pH range, the resulting colonies after incubation were counted. The tolerance of the isolates to acidic pH was detected by comparing the number of cfu/ml before exposure to the acidic pH with the values after subjection.

In vivo studies

Experimental animals

Isolation and enumeration of the microbial flora in the G.I.T. of apparently healthy albino rats were carried out before the experimental animals were randomly assigned to 4 treatments AE, BE, CE and DE of 5 rats each. Treatments AE and BE were
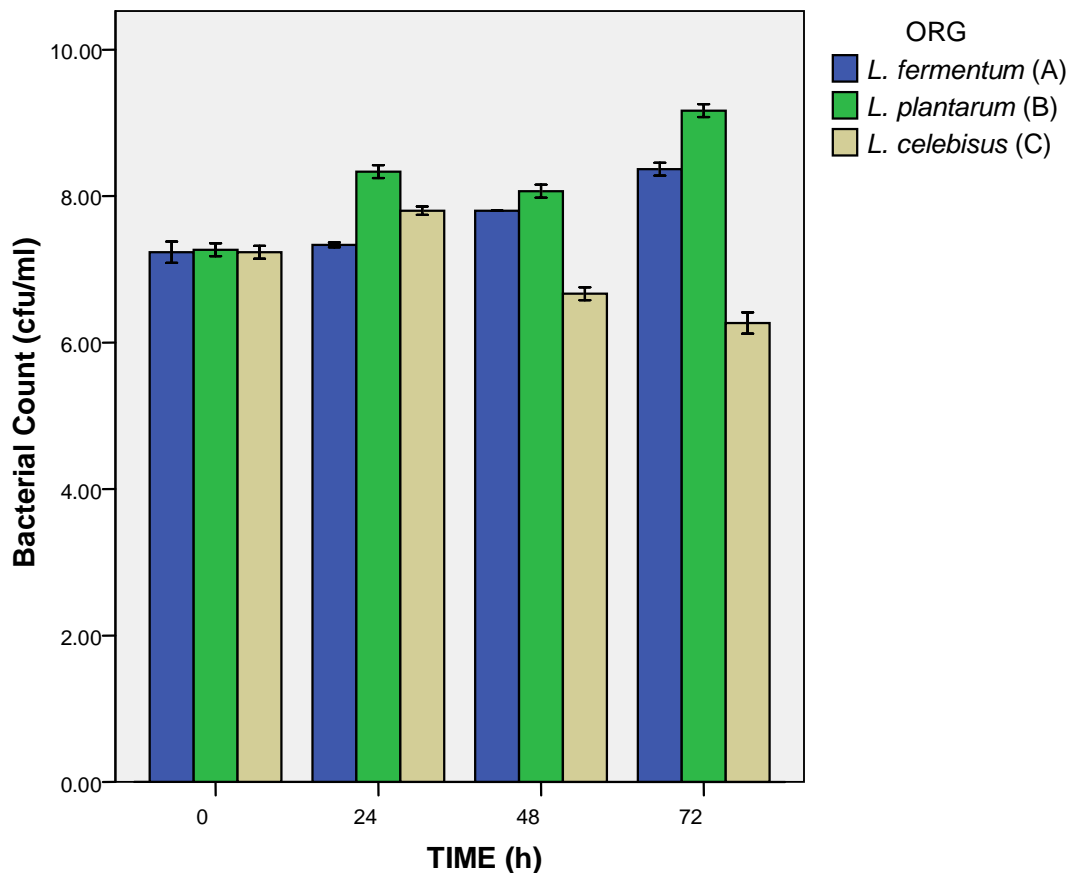
Figure 1. Growth of *L. fermentum*, *L. plantarum* and *L. cellebiosus* in lima bean flour during 72 h fermentation at 37°C (10^6 cfu/ml).

infected with *E. coli* (0.3 ml of 10^5 cfu/ml daily for 3 days) while CE and DE were not infected. After a 4-day post ingestion period was observed, diet of treatments AE and CE were supplemented with 10 g each of the fermented sample for 21 days. After feeding on the experimental diet for 3 weeks, all animals were fed the control (basal diet) for a further 7 days. Thereafter, total weight gain and feacal characteristics (colour and texture) were observed while bacterial enumeration of feacal samples at 7, 10, 14, 28, 35 and 52 days was also determined using conventional techniques. All animals were placed on a basal diet *ad libitum*.

Statistical analysis

Unless otherwise indicated, results are expressed as means ± SD of three replicates. Data were subjected to one–way analysis of variance (ANOVA) using SPSS version 14.0. The Duncan's Multiple Range test was used to separate the means at the 5% level of probability.

RESULTS

The population of *Lactobacillus* species in Lima beans flour increased with the period of fermentation except for *L. cellebiosus* (Figure 1). The bacterial count increased from 7.3×10^6 to 9.15×10^6 in B1 for *L. plantarum*, from

7.35×10^6 to 8.4×10^6 in A1 for *L. fermentum* and decreased from 7.36×10^6 to 6.4×10^6 in C1 for *L. cellebiosus* between 0 and 72 h of fermentation. Also, there was significant increase ($p \leq 0.05$) in bacterial counts in products stored at room temperature (A1, B1and C1) compared to those stored at refrigeration temperature (A2, B2 and C2) (Figure 2).

On the basis of pH, titratable acidity and temperature, the three fermented samples studied showed high similarity (Table 1). There was significant decrease ($p \leq 0.05$) in pH at the end of 72 h fermentation while titratable acidity increased significantly ($p \leq 0.05$) for all the fermented samples.

Table 2 shows the proximate composition of the samples. There was a significant increase in the protein content (27.62 to 34.78) and decrease in carbohydrate, moisture, crude fibre and fat contents in the fermented sample.

Figure 3 shows the mean weight of experimental animals during the 52 day feeding trials. There was decrease in mean weight in Treatment AE (animals placed on basal diet, infected with *E. coli* and then fed with the experimental diet) from 55 g (day 1) to 51 g (day 7) after infection with *E. coli*. There was a drop in weight
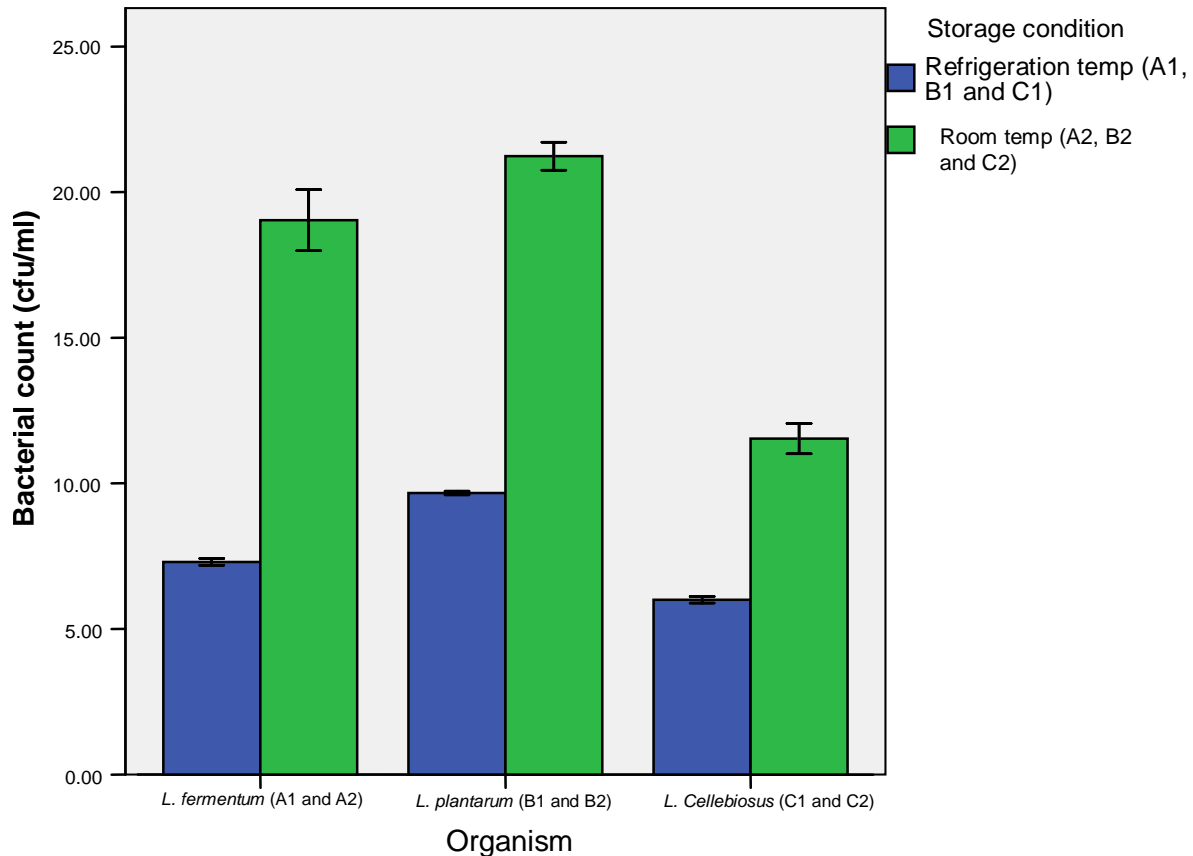
Figure 2. Bacterial counts (10^5 cfu/ml) of *L. fermentum, L. plantarum* and *L. Cellebiosus* in lima beans flour after storage for 14days at refrigeration temperature (4 ± 2°C) and room temperature (24 ± 2°C), respectively.

Table 1. Physiochemical changes during fermentation.

| Time (h)	Isolates	pH*	Titratable* acidity (%)	Temperature*
0	A	6.83 ± 0.01	0.33 ± 0.01	24.10 ± 0.10
	B	6.83 ± 0.01	0.37 ± 0.01	24.00 ± 0.00
	C	6.85 ± 0.005	0.35 ± 0.005	24.03 ± 0.06
24	A	5.42 ± 0.02	1.26 ± 0.005	24.20 ± 0.06
	B	5.20 ± 0.00	1.35 ± 0.00	24.67 ± 0.29
	C	5.48 ± 0.005	0.65 ± 0.00	24.47 ± 0.06
48	A	4.01 ± 0.01	1.59 ± 0.01	25.70 ± 0.06
	B	4.65 ± 0.15	1.73 ± 0.06	25.93 ± 0.58
	C	5.13 ± 0.01	1.13 ± 0.05	24.93 ± 0.11
72	A	3.27 ± 0.005	1.86 ± 0.01	26.40 ± 0.12
	B	3.10 ± 0.005	2.00 ± 0.005	27.03 ± 0.06
	C	4.01 ± 0.00	1.24 ± 0.01	26.17 ± 0.29

*Values are mean of three replicates.

to 48 g (day 10) then a little rise to 53 g (day 14) on consumption of the experimental diet. From day 28 to day 52, there was a steady increase in mean weight from 61 to 76 g. Animals in treatment BE (animals placed on

Table 2. Proximate composition of Lima beans flour before and after fermentation with *L. plantarum.*

Composition (%)	Before fermentation*	After fermentation*
Protein	27.62 ± 0.01	34.78 ± 0.02
Crude fibre	1.95 ± 0.02	1.42 ± 0.01
Carbohydrate	56.25 ± 0.00	50.13 ± 0.01
Fats	2.34 ± 0.01	2.92 ± 0.00
Ash	1.56 ± 0.00	1.21 ± 0.39
Moisture	10.28 ± 0.00	9.54 ± 0.01

*Values are mean of three replicates. Keys: PRO- protein, FIB- crude fibre, CAR- carbohydrate, FTS- fats, ASH- ash and MST- moisture.
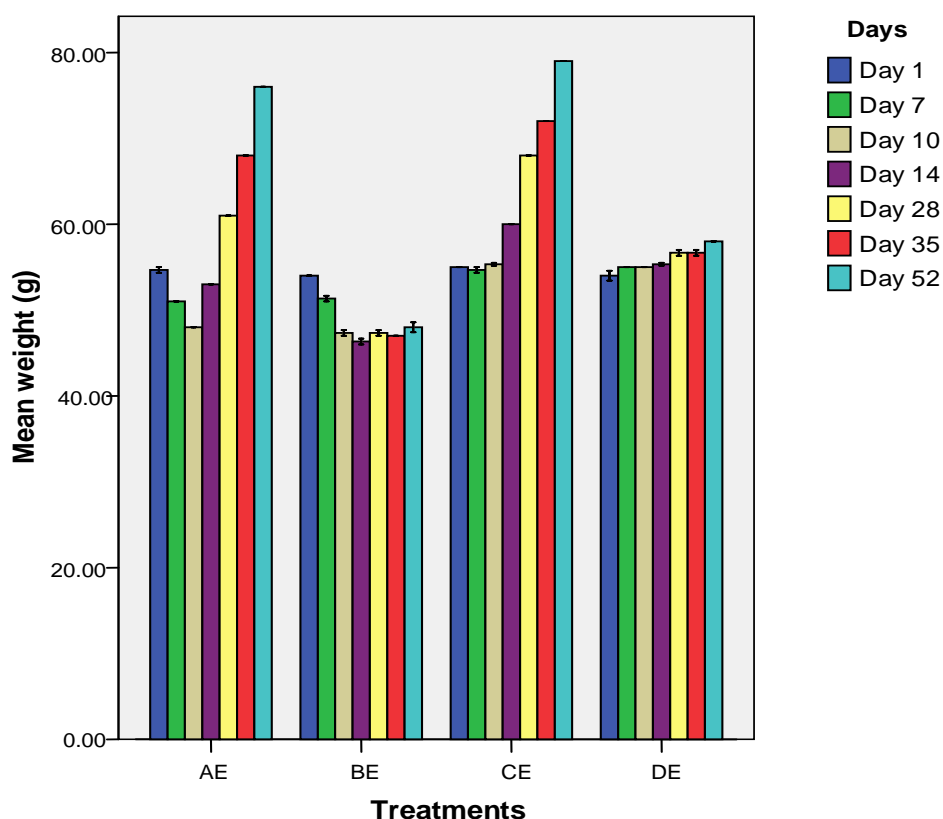
Figure 3. Effect on the growth performance (mean weight) during *in vivo* feeding trials. Keys: AE: Rats infected with *E. coli* and then fed with the experimental diet; BE: Rats infected with *E. coli* alone; CE: Rats fed with the experimental diet alone; DE: Rats placed on basal diet alone (control).

basal diet and infected with *E. coli*) showed decrease in weight from 51 g (day 1) to 46 g (day 14) then a gradual increase in mean weight from day 28 (47 g) to day 52 (50 g). Treatment CE (animals placed on basal diet and fed with the experimental diet) showed steady increase in weight from 55 g (day 7) to 79 g (day 52). Experimental animals fed with basal diet alone (Treatment DE) showed a not too noticeable increase in weight from 54 g (day 1) to 59 g (day 52).

The feacal characteristic of the rats during *in vivo* feeding experiment is shown in Table 3. Rats infected with *E. coli* (BE) had symptoms of diarrhea throughout the feeding period. The results of the microbial analysis of feacal sample are displayed in Figures 4 to 7. After 52 days of feeding, a significant increase (p ≤ 0.05) in the number of lactobacilli was observed in groups fed with the experimental diet (AE and CE) compared with the control groups (BE and DE), while the number of *E. coli* and

Table 3. Feacal characteristics (colour and texture) of rats during *in vivo* feeding trials.

Treatments	Day 1	Day 7	Day 10	Day 14	Day 28	Day 35	Day 52	Remarks
BE	DB/SM	LB/WL	LB/WL	LB/WL	LB/SM	DB^p/SM	DB^P/SM^p	Diarrhea ****
CE	DB/SM	DB/SM	DB/SM	DB/SM	DB/SM	DB/SM	DB/SM	No Diarrhea
AE	DB/SM	LB/SM	LB/WL	LB/SM	DB^P/SM	DB/SM	DB/SM	No diarrhea
DE	DB/SM	DB/SM	DB^p/SM	LB/WL	LB/SM^p	DB/SM^P	DB/SM	No diarrhea *

Keys: BE: Rats infected with *E. coli* alone; CE: Rats fed with the experimental diet alone; AE: Rats infected with *E. coli* and then fed with the experimental diet; DE: Rats placed on basal diet alone (control); DB: Dark brown faeces; LB: Light brown faeces; SM: soft/moist faeces; SM^P: partial soft /moist faeces; WL: wet lose faeces *Number of rats in the treatment that passed out diarrhoeic faeces.
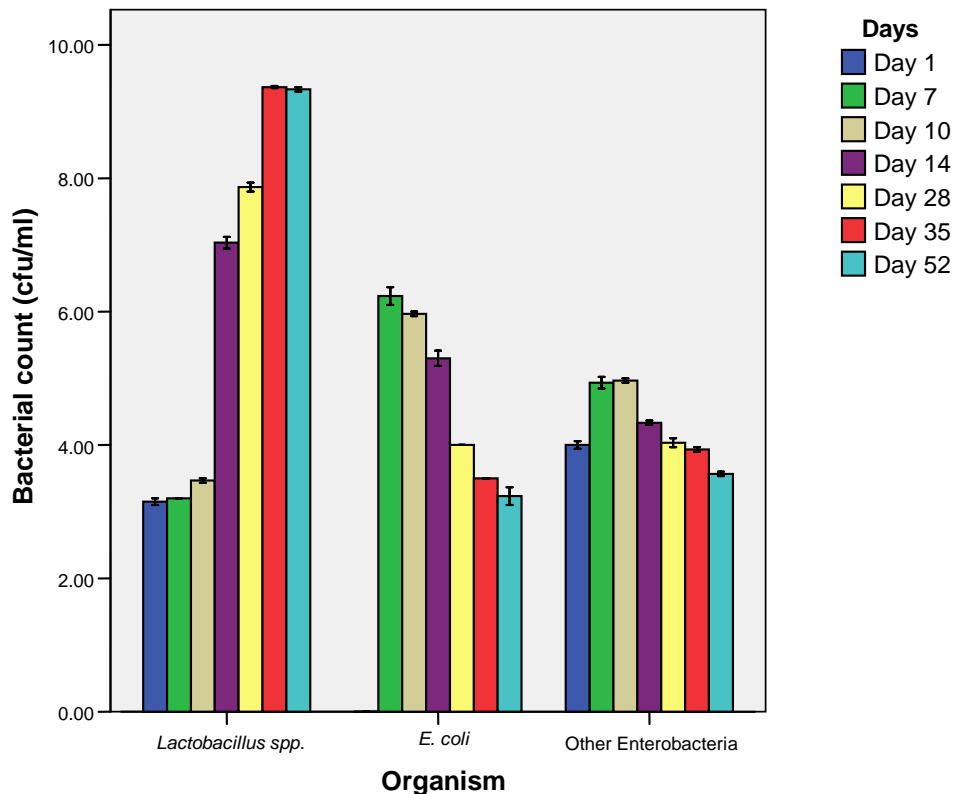
Figure 4. Bacterial counts of treatment AE during *in vivo* feeding trials. Keys: AE: rat infected with *E. coli* and then fed the fermented product, LAB: *Lactobacillus* spp., ECO: *E. coli*, ENT: other feacal enterobacteria.

other Enterobacteria decreased ($p \leq 0.05$) significantly.

DISCUSSION

Various media have been studied as carriers of probiotic bacteria, such as soy, cheese and peanut (Bergamini et al., 2005). To exert their beneficial effects on the host, it is essential that lactic acid bacteria (LAB) be alive in sufficient numbers in the products at the time of consumption (Corgan et al., 2007). It is not sufficient to establish the exact viable count of probiotic bacteria in a functional food in order to insure their health benefits

because this number varies with the strain and food. However, 10^7 cfu/ml of food or feed sample is recommended as the minimal probiotic population required to impact favourably on the consumers health (Bergamini et al., 2005).

L. plantarum having an appreciable increase in cell growth after storage fulfill the criteria as a good probiotic organism. All the products stored at room temperature (24 ± 2°C) for 14 days, A2 (*L. fermentum*), B2 (*L. plantarum*) and C2 (*L. cellebiosus*) had marked increase in cell growth of 19.4 × 10^6, 21.7 × 10^6 and 11.1 × 10^6 cfu/ml respectively compared to those stored at refrigeration temperature (4 ± 2°C). As with all fermented
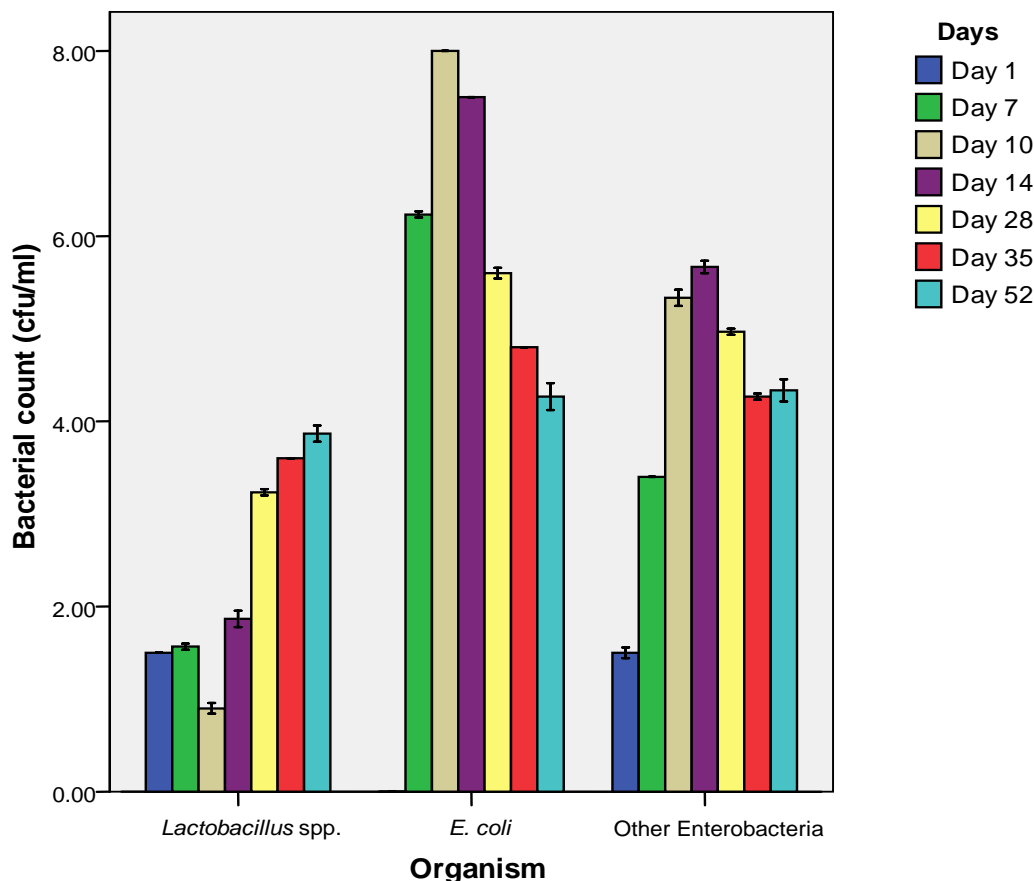
Figure 5. Bacterial counts of treatment BE during *in vivo* feeding trials. Key: BE- Rats infected with *E. coli* alone, LAB: *Lactobacillus* spp., ECO: *E. coli*, ENT: other feacal enterobacteria.

dairy products containing living bacteria, probiotic products must be cooled during storage. This is necessary both to guarantee high survival rates of the probiotic organisms and to ensure sufficient stability of the product (Vasiljevic and Shah, 2008).

The reduction in pH observed in this study has been reported to be due to the production of acids by fermenting microorganisms which did not seem to affect the growth of the *Lactobacillus* species (Dziedzoaze et al., 1996).

The significant increase ($p \leq 0.05$) in protein content (27.62 to 34.78) is probably due to increase in microbial cell mass during fermentation. Increase in protein content of food resulting from increase in microbial cell mass has been reported by other investigators during fermentation of various foods including jack beans (Onyango et al., 2004) and soya products (Ojokoh and Wei, 2011) while the significant decrease ($p \leq 0.05$) in carbohydrate content (56.25 to 50.13) agrees with the findings of Odetokun (2000) who reported that fermentation decreased the carbohydrate content of cereal and legume blend when compared with unfermented samples. The decrease in dry matter in the fermented

sample compared to the unfermented sample might be due to lactic acid accumulation with concomitant increase in acidity and a decrease of dry matter yields. The decrease in ash content may be due to the fact that some biological macromolecules were released into the solution from such structures (Yagoub and Abdalla, 2007). Decrease in ash content has been reported in various foods including sieve maize mash (Antai and Nzeribe, 1992) and garri (Sanni, 1991). The low value of fibre in the fermented sample is desirable because possible undesirable aspects of high fibre levels in weaning period include: increased bulk and lower calorie density, irritation of the gut mucosa, reduced digestibility, reduced vitamin and mineral availability and local effects on intestinal mucosa (Odetokun, 2000). However, the lower moisture content of the fermented sample compared to the unfermented lima bean sample indicates that the product will have a good keeping quality since spoilage microbes thrive better in the presence of adequate moisture.

Probiotics had been used as growth promoters to replace the widely used antibiotic and synthetic chemical feed supplements (Fuller, 1989). This has been possible
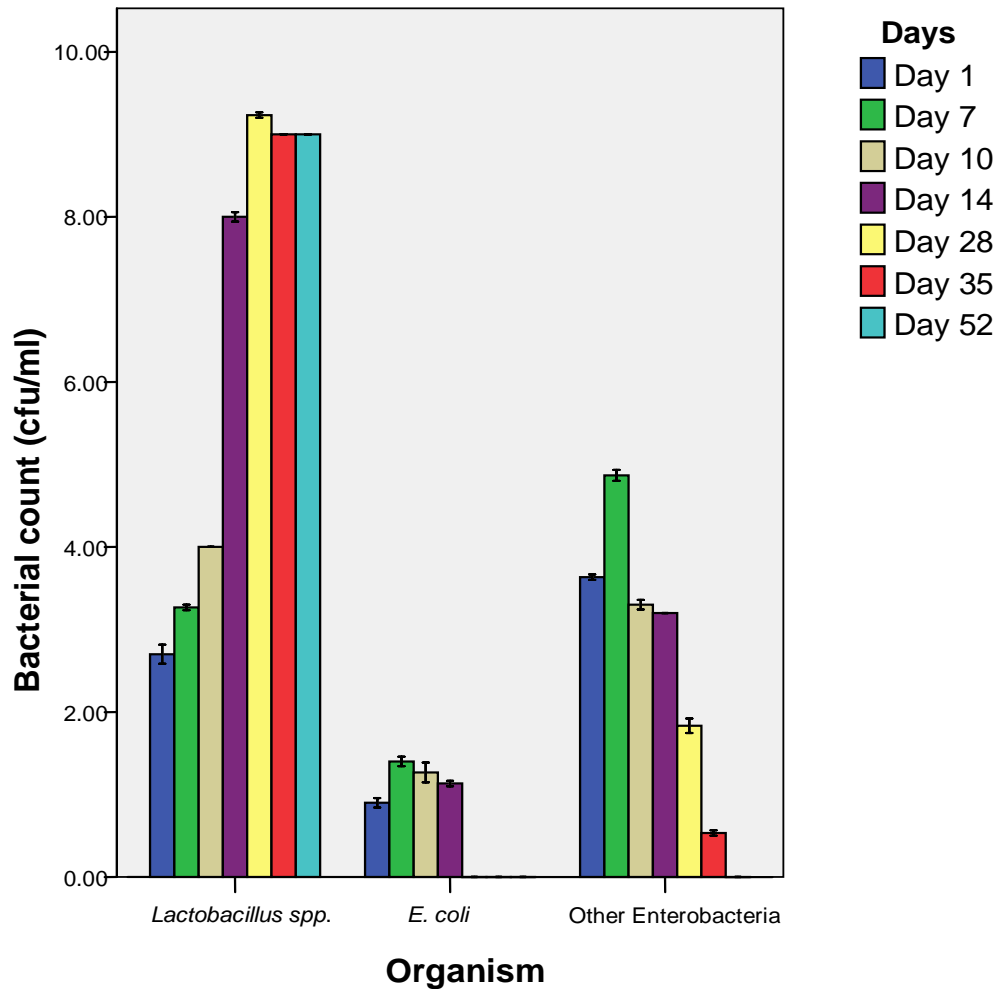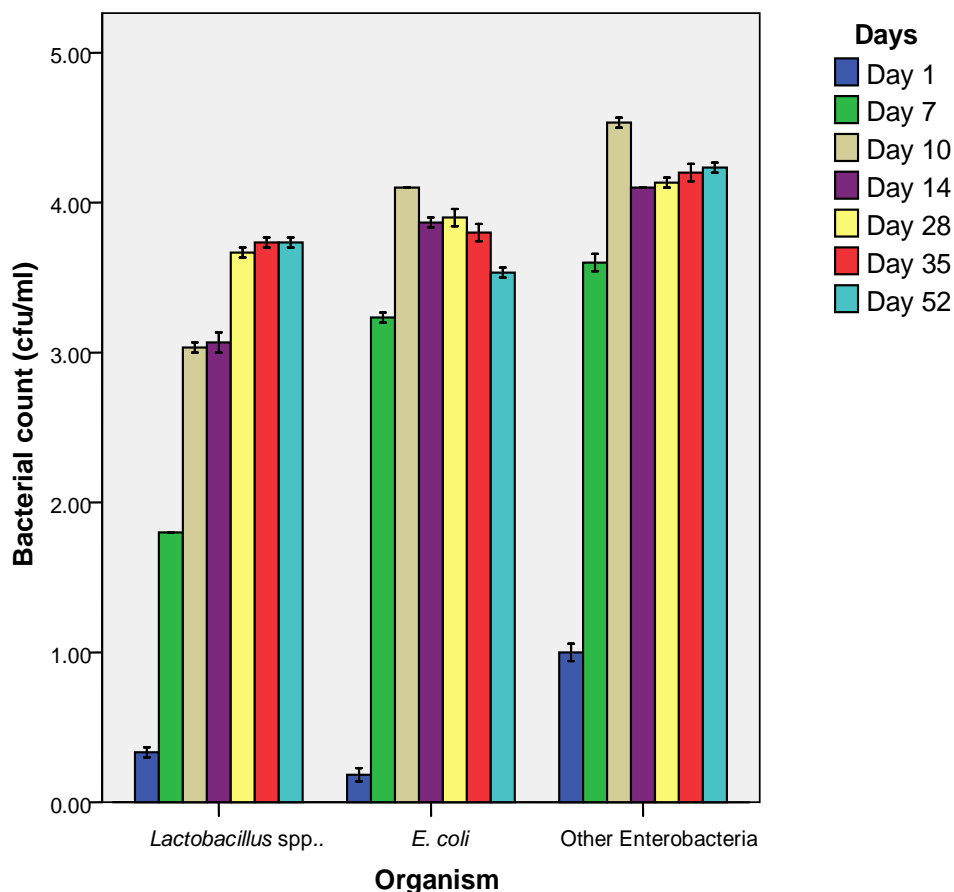
Figure 6. Bacterial counts in treatment CE during *in vivo* feeding trials. Keys: CE- Rats fed with the fermented product alone, LAB: *Lactobacillus* spp., ECO: *E. coli*, ENT: other feacal enterobacteria.

by their ability to inhibit the presence of growth depressing microflora and also by enhancing absorption of nutrients through the production of digestive enzymes. After 52 days of feeding as shown in Figure 3, it was observed that treatments AE (rats infected with *E. coli* and then challenged with the probiotic food) and CE (rats fed with the probiotic food alone) were found to weigh more than treatments BE (rats infected with *E. coli* alone) and DE (rats fed with the basal diet alone). Rats infected with *E. coli* (BE) had symptoms of weakness and wet loose faeces throughout the 52-day feeding trials, whereas in treatment AE, (rats infected with *E. coli* and then fed the experimental diet), such symptoms were initially visible during the first 14 days of infection with *E. coli*, but disappeared rapidly after they were challenged with the probiotic food. There were no diarrheic symptoms in rats fed with the experimental diet (CE) all through the 52 days period of feeding while rats placed on the basal diet alone (DE) showed some symptoms of

diarrhea. A similar observation in pig was also reported where there was a reduction in the prevalence of pig diarrhea during the suckling phase when a probiotic, Cenbiot, compounded from *Bacillus* species was used (Zani et al., 1998). Some probiotic *Lactobacillus* strains have been successfully used in the dairy industry to reduce the incidence of traveller's diarrhea and to promote recovery from acute diarrhoea (Isolauri et al., 1991).

The small number of units of Enterobacteriaceae and other Gram-negative bacteria obtained from the microbial analysis of feacal sample showed that their growth might have been inhibited due to the presence of the *Lactobacillus* species (*L. plantarum*). This agrees with Oyewole (1997) that lactic acid bacteria involved in fermentation of African foods have been documented as having the health benefit of shortening periods of diarrhea. This is also in accordance with findings by Bukowska et al. (1998) who reported that *L. plantarum*

Figure 7. Bacterial counts in treatment DE during *in vivo* feeding trials. Keys: DE- Rats placed on basal diet alone, LAB: *Lactobacillus* spp.*,* ECO: *E. coli*, ENT: other feacal enterobacteria.

increases the level of α-3 unsaturated fatty acids in foods thus protecting such foods from multiplication of harmful, potentially pathogenic bacteria. This may also be a reason for the faster increase in body mass in the experimental animals.

Conclusion

There is therefore considerable evidence that *L. plantarum* showed good growth and survival in lima bean flour and had positive effect on the intestinal microbial balance of albino rats. Thus, lima bean flour fermented with *L. plantarum* could be an ideal probiotic food.

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