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Exploitaton of the Functional Properties of *Leuconostoc mesenteroides* and *Lactobacillus plantarum* as Probiotic using Soyabeans Flour as Vehicle

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Authors' contributions

This work was carried out in collaboration among all authors. Authors AM and MY designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. All the authors managed the analyses of the study. Author SA managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Lactic acid bacteria produce a variety of antimicrobial compounds such as acetic acid, hydrogen peroxide, diacetyl on them as a natural competitive means to overcome other microorganism sharing the same niche. Seven different strains of *Leuconostoc mesenteroides* and *Lactobacillus plantarum* were screened for their ability to produce enzymes and metabolites. They were also screened for their ability to withstand some physiological stress like acid tolerance, temperature, salt concentration and antibacterial activity. *Leuconostoc mesenteroides* S3 and *Lactobacillus plantarum* Yh1produced values significantly different to other five isolates and they were selected. These functional properties were exploited in the fermentation of soyabean in order to obtain a probiotic vehicle. The selected isolates were used as starter both singly and in consortium. This research work also finds out the suitability of soya beans flour as a vehicle for probiotic microorganisms (*Lactobacillus plantarum and Leuconostoc mesenteroides*). The cleaned, dried and roasted soya

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beans were grinded to flour. The soya beans flour was fermented with *Lactobacillus plantarum* and *Leuconostoc mesenteroides* and there was mass increase in lactic acid counts in the fermented soya beans flour store at room temperature. All lactic acid fermented soya beans flour recorded increase at room temperature but slight reduction in number at refrigerator temperature from day 0 to day 14 of storage. Lactobacillus plantarum fermented soya beans flour (LPFSB) recorded increase from $5.9x10^5$ (day 0) to $10.60x10^5$cfu/g (day 14), while the L. plantarum stored at refrigeration temperature had $5.9x10^5$cfu/g on (day 0) to $5.5x10^5$cfu/g on day 14. The L. plantarum and consortium of both starters used were observed to have considerable increase in cell growth after storage, therefore this satisfies the criteria for good probiotic bacteria. *Leuconostoc mesenteroides* fermented soya beans slightly reduced in cell number at pH of 2 but others survived well at acidic pH and 10% bile. The cell number reduced from $4.8x10^6$ (initial) to $4.2x10^6$ (final). The pH of the intestine ranges from 2.5 to 3.5 and the starter culture used survive this pH and the bile product which shows greatly that they can serve as probiotic in the treatment of infection in gastrointestinal tract .

Keywords: Fermented food products; probiotic; soyabeans flour.

1. INTRODUCTION

Globally, varieties of fermented food products are produced, which contribute significantly to the diets of many people [1]. Fermented food products are commonly describe as a unique class of food products characterized by various kinds of carbohydrate and protein breakdown (food conversion) in the presence of probiotic microorganisms, but rarely is carbohydrate the only constituent acted upon [2]. Fermented food and beverage products have emerged as not only the source of nutrition but also as functional and probiotic foods, which besides nutritional value have health effects or provide protection against food-borne diseases.

Soybeans are very rich in nutritive components. Apart from the very high protein content, soybeans contain a lot of fibre and are rich in calcium, magnesium. The soy protein has a high biological value and contains all the essential amino acids. Soybeans are rich in unsaturated fatty acids and low in saturated fatty acids, which need to be avoided, fermentation reduces the level of unsaturated fatty acids and increases that of saturated fatty acids. Soya beans contain lots of micro and macro nutrients for sustainability of both human and probiotic needs, this makes them suitable to be use as vehicle (substrate) for probiotic organisms (Nuf and Sanz, 2013).

Very high percent of the human population has gastrointestinal disorders as a result of a poor diet, stress and unhealthy lifestyle [1]. Probiotics are seen as a strategy to restore composition and function of gut microbiota, which in turn may lead to decrease in gastrointestinal disorders [2,3]. The most significant genera of probiotics commercialized to date are *Lactobacillus* and *Bifidobacterium* [4]. Probiotics may provide a relief from lactose intolerance and prevent episodes of diarrheas of different etiologies [5]. However, evidence for efficacy of existing probiotics in humans is less strong than expected [6], which has encouraged the selection of strains with improved functions from unconventional sources [7].Probiotics are defined as live microorganisms, which when administered in adequate amounts, confer health benefits on the host. Health benefits have mainly been demonstrated for specific probiotic strains of the following genera: *Lactobacillus*, *Bifidobacterium*, *Saccharomyces*, *Enterococcus*, *Streptococcus*, *Pediococcus, Leuconostoc*, *Bacillus* and *Escherichia coli*. The human microbiota is getting a lot of attention today and research has already demonstrated that alteration of this microbiota may have far-reaching consequences. One of the possible routes for correcting dysbiosis is by consuming probiotics [8].

In this study, the aim was to establish the functional properties and probiotic potential of *Leuconostoc mesenteroides* and *Lactobacillus plantarum* using soya beans as a vehicle (substrate).

2. METHODOLOGY

2.1 Source of Organisms

Lactic acid bacteria (LAB) used in this study was isolated from fermented foods and were identified and characterized using conventional methods. The identified lactic acid bacteria were

stocked culture in Microbiology Laboratory of Waziri Umaru Federal Polytechnic, Birnin Kebbi, Kebbi State while the indicator organisms used were collected from Sir-Yahaya Memorial Hospital, Birnin Kebbi, Kebbi State.

2.2 Collection of Sample

Soyabean (*Glycine max*) seeds were purchased from the Central Market, Birni Kebbi, Kebbi State.

2.3 Preparation of Sample

The soyabeans were sorted out for stone, rot and other physical defects. The cleaned beans were dried to obtain about 9.59% moisture content. The beans were roasted on hot- plate until golden brown, the beans were de-hulled immediately after roasting and allow to cool, the roasted beans were milled with Hammer mill (Model GG-300, Henan Gelgoog commercial and Trading co., China) and sieved with 75-micron mesh to obtained the soybean flour [9].

2.3.1 Screening of isolates for physiological stress

Physiological tests were conducted as described by Schlinger and Lucke (1989). The isolates were tested for growth at different pH level, different temperature and different salt concentration. Suspected LAB was tested at pH of 2, 3, 4, 5, 8.5 and 9.6, and at growth temperature of 15^{o}C, 25^{0}C and 45^{o}C.

2.3.2 Screening of isolates for enzymatic activities

The isolates were screened for their ability to produce protease, amylase and lipase enzymes respectively.

2.4 Determination of Proteolytic Activities

The proteolytic activity of the isolates was determined with slight modification of the method described by Katekan *et al.* [10]. For this, a single colony of these bacteria was inoculated on skimmed milk-MRS agar plate for preliminary assay. These were incubated under anaerobic condition after which Zone of inhibition were measured in millimeter using meter rule. In addition, a single colony of these bacteria was inoculated into test tube containing 3 ml of MRS broth. The culture was then grown under anaerobic condition. After incubation, the bacterial cells were harvested at 4,000 rpm for 30 min. The supernatant was collected for further use as the source of the protease enzymes and thus referred to the “crude extract”. For secondary assay, 5 µl of the crude extract were used to spot on a skim milk agar plate. The inoculated plate was then incubated under anaerobic condition. The presence of a clear zone was recorded and used to indicate the bacterial ability to produce proteases.

2.5 Determination of Amylolytic Activity

The procedure of Harrigan and McCance, [11] was used for the determination of amylolytic activity of lactic acid bacteria. Reconstituted MRS with 1% soluble starch agar was sterilized at 121 °C for 15 min, before being poured to set in sterile plates, making single streaks of culture on the dried plates, and incubated at 30 °C for 48hours. After incubation, the plates were flooded with Gram’s iodine. Non hydrolyzed starch formed a blue coloration with iodine, while the clear zone around the growth region indicated starch hydrolysis.

2.6 Determination of Lipolytic Activity

Spirit Blue Agar (SBA) medium was used to screen for lipase-producing LAB. SBA medium was supplemented with 20% olive oil and homogenized to give a stable emulsion, and then autoclaved at 121 °C for 15 min. The sterile medium was poured into sterile Petri-dishes and allowed to set. Then 0.6 cm diameter holes were bored under aseptic conditions using a sterile cork-borer. The test was performed by inoculating 0.2 ml of the cells of a 24hours broth culture, obtained by centrifugation at 5000 rpm into the wells. Lipolytic ability was detected on this medium as a blue zone around the inoculated wells. All plates used were incubated at 30 °C for 48 h. An un-inoculated control was also performed [12].

2.6.1 Quantitative determination of antimicrobial compound produced by lactic acid bacteria

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

2.6.2 Quantitative estimation of lactic acid production

The production of lactic acid was determined by transferring 25ml of supernatant fluid of test organisms into 100ml flasks. This was titrated

with 0.1M NaOH and 1ml of phenolphthalein indicator (0.5 in 5% alcohols). The titratable acidity was calculated as lactic acid % w/v [12]. Each millimetre of 0.1M NaOH is equivalent to 90.08mg of Lactic acid. The titratable acidity was then calculated as stated in A.O.A.C [13] as given below:

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

2.6.3 Quantitative estimation of hydrogen peroxide production

20ml of dilute H_2SO_4 acid was added to 25ml of the supernatant fluid of the test organism. Titration was carried out with 0.1M potassium permanganate (KMnO4). Each ml of 0.1MPotassium permanganate is equivalent to 1.79mg of Hydrogen peroxide solution. Decolourization of the sample was regarded as the end point. The volume of H_2O_2 produced was then calculated [13] as given below:

Where; Ml $_{KMnO4}$ = Volume of $KMnO_4$ used, N $KMnO_{4=}$ Molarity of $KMnO_4$ solution, ml H_2SO_4 = Volume of H_2SO_4 added, M.E = Equivalent factor.

2.6.4 Quantitative estimation of diacetyl production

Diacetyl production was determined by transferring 25mL of the supernatant fluid of the test organisms into 100mL flasks. Hydroxylamine solution (7.5 ml) of 1M was added to the flask and to a similar flask for residual titration. Both were titrated with 0.1M HCL to a greenish yellow end point using bromophenol blue as indicator [12] and the equivalent factor of HCL to diacetyl is 21.52mg. The concentration of diacetyl produced was calculated using the A.O.A.C [13] as given below:

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

2.7 Evaluation of Antagonistic Activity

Agar well was used to confirm the antagonistic activities of the (LAB) isolates. The methods of (Yusra *et al.* 2013) [14] were used to determine the antibacterial activities of the (LAB) isolates. Single isolated colonies were selected from (MRS) agar plates and transferred to grow in sterile (MRS) broth. The broth culture was incubated at 37^{o}C for 48 hours. After incubation, the culture was centrifuged at 4,000 rpm for 30 minutes to obtain the culture supernatant. The indicator microorganisms (*Escherichia coli, Salmonella typhii, Bacillus subtilis, Shigella* spp*, Pseudomonas aeruginosa, and Klebsiella* spp) were grown in nutrient broth for 24 hours at 37^{o}C. A sterile cotton swab was dipped into the culture of the indicator microorganisms and rotated several times and the swab was then pressed firmly on the inside wall of the tube above the fluid level to remove excess inoculums. The dried surface of sterile Mueller Hinton Agar plates was inoculated with the indicator microorganisms by streaking the swab over the entire agar surface. This procedure was repeated by streaking two or more times while rotating the plate each time to ensure even distribution of inoculums. 100 ml of cell free supernatants was filled in 8-mm diameter sealed wells cut in the Mueller Hinton Agar already inoculated with the indicator microorganisms. The inoculated plates were incubated for 24 hours at 37 °C, and the diameter of the inhibition zone was measured by metre rule in millimeters.

2.8 Inocula Development

Species of lactic acid bacteria (*Leuconstoc mesenteroides* and *Lactobacillus plantarum*) obtained were developed. Pure cultures of the organism were inoculated in sterile saline suspension to make 0.5 McFarland turbidity standards which would be used as inoculums.

2.9 Fermentation and Storage

Soya beans flour were mixed with distilled water (1:3) in 25ml fermentation jars which were autoclaved at 121°C for 15 min. Jars were allowed to cool after which each jar were inoculated with the starter culture both singly and in consortium in duplicates and one 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 [15]. After, fermentation the fermented bean was hot air dried and store at 25 ± 2°C (room temperature) for 14 days. Viable counts of (LAB) in the products were determined during the period of fermentation and after storage.

2.9.1 *In vitro* studies of gastrointestinal tolerance

Starter culture tolerance to different acidic conditions in the fermented food was tested by centrifuging the fermented food containing the starter 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). One milliliter of this dilution (pellet in saline solution) was plated for each of the fermented samples; this was done so as to estimate the number of viable cells that will be subjected to the acidic pH. Nine milliliter of sterile distilled water that had already been adjusted to pH 2, 3, 4 and 5, using phosphate buffer saline was transferred into already labeled test tubes, which will be done in triplicate for each sample. Then, 1ml of the re-suspended pellet containing the isolates was inoculated into the appropriate test tubes; this was shaken and incubated at 37°C for 3 h. After three hours of incubation, the appropriate dilutions was plated on De Mann Rogosa and Sharpe Agar and incubated anaerobically at 37°C for 54h. 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/g before exposure to the acidic pH with the values after subjection. Also, survival in bile was done by inoculating test isolates into broth containing 10 % of bile which was incubated overnight at 37°C. Then, 1 ml of this culture was plated on MRS agar and incubated for 54hrs at 37°C; survival in bile was taken as growth on the plates [16].

3. RESULTS AND DISCUSSION

The lactic acid bacteria were screened for their amylase, protease and lipase production. Results showed that, out of 7 isolates tested, 2 isolates representing approximately 25% could produce the three enzymes. However, it was also evidence that these bacteria had different enzyme activity; *Lactobacillus plantarum*Yh1 produce the highest protease and amylase while the highest lipase activity was produced by *Leuconostoc mesenteroides* S3. To group these enzyme-producing bacteria, the size of the diameter of the clear zone was used as an indicator as illustrated in Fig. 1. However, all the isolates tested showed at least one enzyme activity. Only two isolates did not show proteolytic activity on skimmed milk agar. Uaboi-Egbenni *et al.* [17] show that *Lactococcus raffinolactus, Pediococcus pentosaceus, Leuconostoc mesenteroides, Lactobacillus brevis, Lactobacillus plantarum, Pediococcus sp. and Pediococcus halophilus* were involved in enzyme production during food fermentation. Several workers have made mention of the presence of *Leuconostoc mesenteroides* m888 during food fermentation because of their ability to produce the three enzymes.

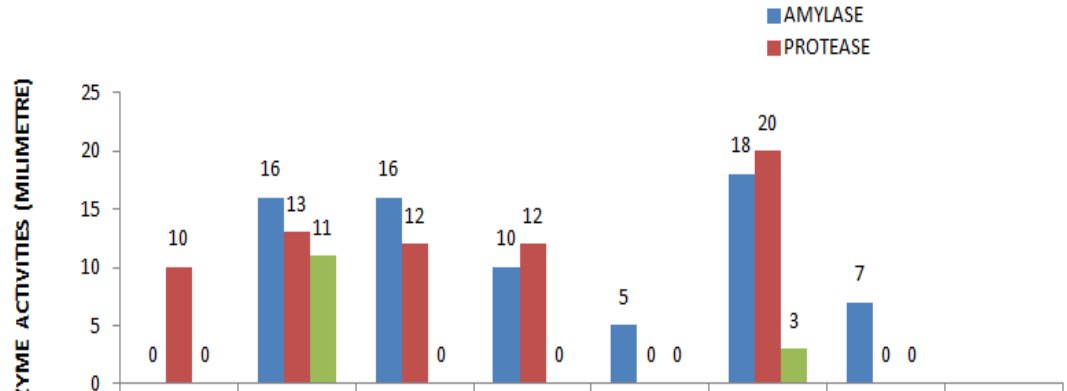
As shown in Fig. 2, at the end of the storage period, there was increase in the lactic acid bacteria counts in the fermented soya beans flour stored at room temperature in all the starter based sample both singly and in consortium. All lactic acid fermented soya beans flour recorded increase at room temperature but slight reduction in number at refrigeration temperature from day 0 to day 14 of storage. *Lactobacillus plantarum* fermented soya beans flour (LPFSB) recorded increase from 5.90 x 10 5 (day 0) to 10.60 x 10^5cfu/g (day 14) compared to the one stored at refrigeration temperature which had 5.90 x 10^5cfu/g on (day 0) to 5.50x10^5 (day 14) cfu/g. According to Gao *et al.* (2010), for lactic acid bacteria to exert their probiotic effects on their host, they should be present in sufficient numbers in the vehicle as at the time of consumption. Also, the number of viable probiotic organism needed to confer benefit on the host varies greatly with the food type and strain of the probiotic organism. According to Anthony *et al.* [16] a viable count of 10^7cfu/g of the bacteria has been recommended as the minimal population of probiotics necessary to give a noticeable effect on the host health. The Soya beans flour fermented with *L. plantarum* and *Leuconostoc mesenteroides* stored at room temperature for 14 days had a significant increase in the bacterial growth which recorded 22.80x10^6cfu/g at room temperature compared to the ones stored at refrigeration temperature (17.60 x10^6cfu/g) at day 14. The *L. plantarum* and consortium of both starters used were observed to have considerable increase in cell growth after storage, therefore this satisfies the criterion for good probiotic bacteria. However, to guarantee high survival rate of the probiotic bacteria with the sufficient stability of the vehicle, the probiotic product must be cool during storage [18]. The viability of these starter culture after 14 days' storage at refrigeration temperature shows that soya beans flour may be a good vehicle for this probiotic bacterium. This has been observed earlier by Anthony *et al.* [16] in their investigation of mucuna beans flour fermented with *Lactobacillus plantarum*.

Several factors have been claimed to affect the viability of probiotic bacteria in dairy foods such as yogurt and fermented milks, including low pH and bile salts. In order to be used as potential probiotics, dairy lactic acid bacteria (LAB) strains need to be screened for their capacity of transit tolerance to the upper gastrointestinal tract conditions (Chou and Weimer 1999). The low pH is known to provide an effective barrier against

the entry of bacteria into the intestinal tract. The pH of the stomach generally ranges from pH 2.5 to pH 3.5 (Holzapfel *et al*., 1998).

Fig 3 shows the tolerance of lactic acid bacteria used as starter culture to acidic pH condition and 10% bile. Both starter cultures survive well at acidic pH and 10% bile except at pH of 2 in which there is slight reduction in cell number observed when *Leuconostoc mesenteroides* ferment soya beans flour. The cell number reduces from $4.8x10^6$ to $4.2x10^6$. The viability and survival of probiotic bacteria are the most important parameters for providing therapeutic functions. Starter culture used *Lactobacillus plantarum* and *Leuconostoc mesenteroides* survive this pH and the bile salt which shows greatly that they can serve as probiotic with the treatment of infection in gastrointestinal tract.

The results in Table 1 were presented in means±standard deviation. The superscripts indicate the ranking of the post hoc test using Duncan Multiple Range Test. The means with different superscripts were significantly different from one another down the column while those with the same superscripts were similar. The asterisks also indicate an experimental set up with significantly different outcomes from the Analysis of Variance (ANOVA).



	Lactobacillus plantarum Yh2	Leuconostoc mesenteroides S3	Leuconostoc mesenteroides S2	Lactobacillus plantarum MG2	Leuconostoc mesenteroides S1	Lactobacillus plantarum Yh1	Lactobacillus plantarum MG1
AMYLASE	0	16	16	10	5	18	7
PROTEASE	10	13	12	12	0	20	0
LIPASE	0	11	0	0	0	3	0

Fig. 1. Enzyme production as depicted by clearance zones (millimetre) during screening on solid medium

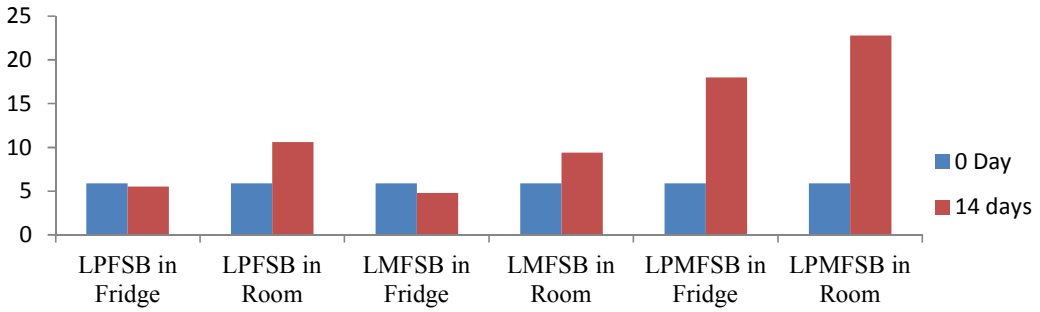


Fig. 2. Lactobacillus count (x 10^6 cfu/g) in fermented soya bean flour after 14 days of storage at different conditions (refrigeration and room temperature)

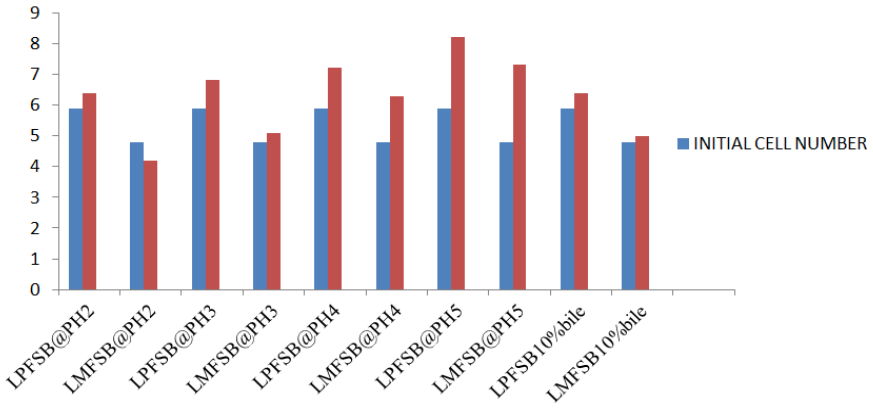


Fig. 3. Tolerance of lactic acid bacteria in fermented soy bean flour to different acidic pH range and 10% bile (x10^{6}cfu/g)

Key: LPSB= Lactobacillus plantarum fermented soya flour; LMSB = Leuconostoc mesenteroides fermented soya flour; LPMSB= Lactocbacillus plantarum and Leuconostoc mesenteroides fermented soya flour and SFSB spontaneously fermented soya flour

The rates of LAB's survival in the fermenting soya bean flour at different fermentation period were tested for significant difference and identification of the particular details of the fermentation time that accounted for the difference observed. During the control experiment, at zero hour of fermentation, the survival rate of LAB was significantly different ($p<0.05$) among the compared highest survival rate was observed with SFSB while the least survival rate was in the LPSB experimental set up. The survival rate of the LAB in LPSB, LMSB and LPMSB were ranked similarly by the Duncan multiple range tests. This implies that the rate of survival in the three soya milk products were similar. The survival rate of LAB across the four different fermenting Soya bean at each of the varied fermentation time were significantly different. The trend however showed that LPMSB and SFSB had the least survival rate $1.0\pm0.40\times10^3$ CFU/ml and $1.2\pm0.90\times10^5$ CFU/ml respectively at the 18th hr of the fermentation while (LPSB) and (LMSB) yielded a survival rate higher than LPMSB and SFSB. Generally, from the outcome of the survival rate was highest $1.8\pm0.21\times10^7$CFU/ml at the 72nd hr fermentation period of LPMSB.

The rate of survival may have differed significantly because of the likelihood of different level of acid tolerance of each of the microorganisms used as starter. The pattern and rate of survival through 72hrs also indicates that LPSB and LMSB were better used separately than combined as starter culture. The rate at which the two species independently modify the fermentation of soya beans was reported to be less likely to favor the survival of the two if they were combined as starter cultures. Ari *et al.* [19] suggested the higher chance of one of the starters to attain exponential growth before the other. This therefore may yield a population of one of the two starters to reach its stationary or pre-decline phase faster than the other if in a consortium as starter cultures. The faster of the two was reported to be *Leuconostoc mesenteroides*, hence the chance of the toxic and starvation-triggered metabolic products at this stage may further affect the survival of the other bacterium (*Lactobacillus plantarum*) in the fermentation broth of Soya Beans. At this stage of fermentation, the culture system approaches a Quassi-State (Equilibrium) where the starter culture with faster nutrient utilization and metabolic adaptation which may result in the selective growth condition created by the fermentation broth if the starters were combined [20].

The least survival of LABs as starter culture was reported in the spontaneous fermentation experiment of Soya bean flour. This was not unexpected since the depletion of nutrient and complex progression of growth phase may result in quick exhaustion of the nutrients which may retard the growth of Lactic Acid Bacteria due to the lack of uncontrolled acidic growth environment. Given the outcome as statistically analyzed and presented above, *Lactobacillus plantarum* was better favored by the fermentation than *Leuconostoc mesenteroides.*

Table 1. Survival of lactic acid bacteria (CFU/ml) in the fermenting soya bean flour at different Fermentation period

Product code	Fermentation hour				
	0hrs*	18hrs*	36hrs	54hrs	72hrs
LPSB	$(1.4\pm0.32\times10^{4})^{a}$	$(3.9\pm0.43\times10^{5})^{c}$	$(4.6\pm0.55\times10^{5})^{c}$	$(6.2\pm0.32\times10^{5})^{c}$	$(5.9\pm0.39\times10^{6})^{c}$
LMSB	$(1.5\pm0.07\times10^{4})^{a}$	$(4.2\pm1.06\times10^{5})^{d}$	$(5.7\pm1.00\times10^{5})^{d}$	$(5.4\pm1.02\times10^{5})^{b}$	$(4.8\pm0.70\times10^{6})^{b}$
LPMSB	$(1.5\pm0.00\times10^{4})^{a}$	$(1.0\pm0.40\times10^{3})^{a}$	$(1.4\pm0.80\times10^{5})^{a}$	$(1.5\pm0.80\times10^{5})^{a}$	$(1.8\pm0.21\times10^{7})^{d}$
SFSB	$(2.5\pm0.50\times10^{5})^{b}$	$(1.2\pm0.90\times10^{5})^{b}$	$(2.4\pm0.03\times10^{5})^{b}$	$(1.4\pm0.21\times10^{5})^{a}$	$(1.0\pm0.20\times10^{6})^{a}$

Key: LPSB= Lactobacillus plantarum fermented soya flour; LMSB = Leuconostoc mesenteroides fermented soya flour; LPMSB= Lactocbacillus plantarum and Leuconostoc mesenteroides fermented soya flour and SFSB spontaneously fermented soya flour. The numbers indicate different hours of fermentation

Table 2. Effect of starter culture on the total bacteria counts (CFU/ml) at different fermentation period

Product code	Fermentation hour				
	0hrs	18hrs*	36hrs	54hrs*	72hrs*
LPSB	$(3.0\pm0.33\times10^{5})^{a}$	$(3.5\pm0.79\times10^{4})^{c}$	$(2.9\pm0.28\times10^{3})^{b}$	$(1.8\pm0.04\times10^{2})^{a}$	$(1.5\pm0.20\times10^{2})^{c}$
LMSB	$(3.1\pm0.64\times10^{5})^{a}$	$(4.1\pm0.55\times10^{3})^{b}$	$(3.1\pm0.48\times10^{2})^{a}$	$(3.0\pm0.51\times10^{2})^{b}$	$(2.4\pm0.10\times10^{2})^{b}$
LPMSB	$(3.0\pm0.70\times10^{5})^{a}$	$(2.0\pm0.61\times10^{3})^{a}$	$(3.2\pm0.03\times10^{2})^{a}$	$(1.6\pm0.90\times10^{2})^{a}$	$(4.1\pm0.47\times10^{1})^{a}$
SFSB	$(3.0\pm0.11\times10^{5})^{a}$	$(2.2\pm0.42\times10^{4})^{c}$	$(2.5\pm0.08\times10^{4})^{c}$	$(1.2\pm0.00\times10^{5})^{c}$	$(4.0\pm0.31\times10^{5})^{d}$

Key: LPSB= Lactobacillus plantarum fermented soya flour; LMSB = Leuconostoc mesenteroides fermented soya flour; LPMSB= Lactocbacillus plantarum and Leuconostoc mesenteroides fermented soya flour and SFSB spontaneously fermented soya flour. The numbers indicate different hours of fermentation

The results in Table 2 were presented in means±standard deviation. The superscripts indicate the ranking of the post hoc test using Duncan Multiple Range Test. The means with different superscripts were significantly different from one another down the column while those with the same superscripts were similar. The asterisks also indicate an experimental set up with significantly different outcomes from the Analysis of Variance (ANOVA).

Initial survival rate was found to be similar and were ranked in the same subset in the post hoc test conducted which means that there was no significant difference in the survival rate at zero hour of the four soya bean products that were inoculated with LAB. However, this was not the case from 18^{th}hr through 72^{nd} hour of fermentation as the survival rate were significantly different ($P<0.05$) when the four fermenting soya beans were compared. At the 18^{th} hour of fermentation, LPSB had the highest number of Bacteria counts while that of LPMSB was the least bacterial count. Apart from the Bacteria count from (SFSB), the other three products had a steady reduction trend from the 0^{th} through 72^{nd} hour of fermentation.

The controlled fermentation condition provided by the combined (LPSB) and (LMSB) yielded the highest bacteria growth which was close to that yielded by the uncontrolled spontaneous fermentation set up. The vitamins and growth factors synthesized and released by the two starter cultures may have influenced the supply of nutrients and spur a faster and better adapted bacterial consortium for survival in the fermentation conducted. This was similar to the reported outcome by Boumerdassi *et al.* [21] that the complex in-situ growth may have favored the growth of acido-tolerant bacteria community which may be useful in probiotic capability of (LABS) when consumed. However, the growth was reported to be healthier for consumption if the fermentation time was optimized for log phase so as not to put the starters in a stress condition that could lead to toxic metabolism and release of decline phase proteins which may result in compromised consumer [22].

The results in the table above were presented in means±standard deviation. The superscripts indicate the ranking of the post hoc test using Duncan Multiple Range Test. The means with different superscripts were significantly different from one another down the column while those with the same superscripts were similar. The asterisks also indicate an experimental set up with significantly different outcomes from the Analysis of Variance (ANOVA).

The concentration of diacetyl produced by the seven selected LAB isolates were significantly different ($p<0.05$). A noticeable trend was observed in the continuous reduction in diacetyl concentration produced by all the LAB isolates as the fermentation hour increased from 8hrs through 24hrs except in that produces by Isolate *Leuconostoc mesenteroides* S2 with highest diacetyle produced at 16^{th} hrs.

The Optimum fermentation period for the production of diacetyle for all the Isolates was 24 hrs except for S3. This was probably associated with the microbial physiology of most LABs [23]. They were reported to yield functional metabolites at an increasing level provided the precursors are available for assimilation by the starter cultures individually [24].

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The Quantity of Lactic acid produced was at overall highest (2.26±0.81) g/L at the 24^{th} hour fermentation using the LAB isolate Yh1. The increasing order of Lactic acid concentration produced by the LAB Isolate was 8hrs< 16hrs<24hrs, only S1 had a higher Lactic acid production at 16^{th}hr than 24^{th}hr fermentation. At each of the hour of fermentation, the Lactic acid production was significantly different among the seven selected and compared Isolates of LAB.

The fermentation trend of Lactic acid production with respect to time was observed to be influenced by period of fermentation as described in [25] where a longer period of fermentation was linked with the rate of Lactic acid production of LABs used in that experiment. However, only *Lactobacillus plantarum* strains were tested in common with this study. But the direct proportion of Lactic acid production and period of fermentation were similar to the findings of both studies. The 24^{th} hour fermentation time was optimum for the Lactic acid production of most tested LABS. This may be due to the favorable

condition at that period for the maximum absorption and the utilization of the glucose and other secondary carbohydrate molecules in the nutrient broth into lactic acid which was present in the solution as lactate [26].

The results in the table above were presented in means±standard deviation. The superscripts indicate the ranking of the post hoc test using Duncan Multiple Range Test. The means with different superscripts were significantly different from one another down the column while those with the same superscripts were similar. The asterisks also indicate an experimental set up with significantly different outcomes from the Analysis of Variance (ANOVA).

The Quantity of H_2O_2 produced by the seven selected isolates were highest at the 24t hour of fermentation except for *Leuconostoc mesenteroides S3* with a 0.03g/L decline in H_2O_2 quantity after refng the peak i.e. the overall peak for the experiment (2.27±0.10) at 16th hour. The measured quantity of hydrogen peroxide was found to significantly differ ($p<0.05$) from one isolate to another at the varied time of fermentation. The increasing order of hydrogen peroxide's production with respect to time of fermentation was 8hrs<16hrs<24hrs except the trend noticed in S3 which was 8hrs<24hrs<16hrs. The least quantity of hydrogen peroxide was yielded by the 8 hrs microbial metabolism of MG1.

Notably, the level of Hydrogen peroxide increased in the same directly proportional pattern with diacetyl, and lactic acid previously reported above. The metabolism of carbon sources by lactate oxidase (enzyme encoded in the genes of the six strains of the test LABs) may be enhanced when fermentation time is lengthened beyond 12hrs [24] time of metabolism was increased from 8 through 24hrs of fermentation. The benefits derivable from the anti-inflamatory, homeostatsis among others if the fermented soya bean product is consumed especially when there is no current immune-suppression condition in the consumer [27].

The results in the table above were presented in means±standard deviation. The superscripts indicate the ranking of the post hoc test using Duncan Multiple Range Test. The means with different superscripts were significantly different from one another down the column while those with the same superscripts were similar. The asterisks also indicate an experimental set up with significantly different outcomes from the Analysis of Variance (ANOVA).

The antimicrobial activity of MG1 yielded the largest inhibitory zone against Shigella spp. Although the range of antimicrobial activity against *E. coli* was between (6.00±0.51) mm and (14.10±2.00) mm. When the isolates were compared in Antimicrobial activity against *E. coli*,

Table 3. Quantity of diacetyl produced by isolates of lactic acid bacteria (g/l)

Isolates	Fermentation Time (hours)/Diacetyl Concentration (g/L)		
	8*	16*	24*
*Lactobacillus plantarum*MG1	0.49±0.10^{b}	1.32±0.00^{b}	1.49±0.20^{a}
*Lactobacillus plantarum*Yh1	0.52±0.11^{c}	1.44±0.18^{c}	2.90±0.53^{d}
Lactobacillus plantarum MG2	0.42±0.02^{a}	1.31±0.71^{b}	1.78±0.06^{b}
*Lactobacillus plantarum*Yh2	0.43±0.01^{a}	1.23±0.50^{a}	1.90±0.73^{b}
*Leuconostoc mesenteroides*S2	0.49±0.34^{b}	1.32±0.05^{b}	2.30±0.12^{cd}
*Leuconostoc mesenteroides*S3	0.45±0.16^{ab}	1.88±0.33^{d}	1.83±0.48^{b}
*Leuconostoc mesenteroides*S1	0.52±0.01^{c}	1.34±0.00^{b}	1.93±0.92^{b}

Table 4. Quantity of lactic acid produced by isolates of lactic acid bacteria (g/l)

Isolates	Fermentation Time (hours)/Lactic acid Concentration (g/L)		
	8*	16*	24*
*Lactobacillus plantarum*Yh2	0.41±0.02^{a}	1.33±0.65^{b}	1.48±0.03^{a}
*Lactobacillus plantarum*Yh1	1.22±0.42^{c}	1.84±0.32^{c}	2.26±0.81^{c}
Lactobacillus plantarum MG1	0.41±0.12^{a}	1.22±0.04^{b}	1.35±0.00^{a}
*Lactobacillus plantarum*MG2	0.49±0.01^{b}	1.53±0.06^{bc}	1.71±0.04^{b}
*Leuconostoc mesenteroides*S2	0.40±0.05^{a}	0.82±0.10^{a}	1.17±0.30^{a}
*Leuconostoc mesenteroides*S1	1.87±0.12^{d}	1.97±0.19^{c}	1.71±0.70^{b}
*Leuconostoc mesenteroides*S3	0.97±0.03^{bc}	1.37±0.07^{b}	2.25±0.16^{c}

Table 5. Quantity of hydrogen peroxide (H_2O_2) produced by isolates of lactic acid bacteria (g/l)

Isolates	Fermentation Time (hours)/H_2O_2 Concentration (g/L)		
	8*	16*	24*
*Lactobacillus plantarum*MG1	0.43±0.010 [a]	1.20±0.95 [b]	1.57±0.77 [ab]
*Lactobacillus plantarum*Yh1	0.92±0.72 [ab]	1.24±0.22 [b]	1.36±0.60 [a]
Lactobacillus plantarum MG2	0.45±0.83 [a]	0.92±0.11 [a]	1.11±0.54 [a]
*Lactobacillus plantarum*Yh2	0.59±0.19 [a]	1.33±0.22 [b]	1.84±0.00 [b]
*Leuconostoc mesenteroides*S2	0.48±0.20 [a]	1.22±0.06 [b]	1.34±0.31 [a]
*Leuconostoc mesenteroides*S3	1.17±0.44 [b]	2.27±0.10 [c]	2.24±0.30 [c]
*Leuconostoc mesenteroides*S1	0.47±0.06 [a]	0.97±0.13 [a]	1.34±0.00 [a]

Table 6. Antibacterial activity (mm) of LAB isolates against different indicator microorganisms

Isolates	Indicator Organism/ Method/ Zone of Inhibition (mm)					
	Escherichia coli*	***Pseudomonas aeruginosa****	***Shigella*spp***	***Klebsiella*spp***	***Bacillus subtilis****	***Salmonella typhi****
*Lactobacillus plantarum*MG1	9.30±1.00[b]	9.1±0.83[b]	9.40±0.23[ab]	9.00±2.12[c]	5.04±0.08[ab]	8.07±0.49[b]
*Lactobacillus plantarum*Yh1	13.10±3.03[d]	9.55±0.00[b]	11.42±0.00[c]	10.21±0.00[cd]	7.00±0.70[c]	9.00±0.12[c]
Lactobacillus plantarum MG2	7.04±0.99[a]	9.10±0.73[b]	8.30±1.05[a]	9.00±2.01[c]	8.60±2.02[d]	8.00±0.66[b]
*Lactobacillus plantarum*Yh2	6.00±0.51[a]	7.00±0.30[a]	7.10±0.50[a]	5.00±0.06 [a]	6.00±0.44[b]	5.00±1.04[a]
*Leuconostoc mesenteroides*S2	11.60±1.47[c]	9.00±0.80[b]	10.11±1.07[b]	12.40±0.55[d]	6.07±1.11[b]	8.00±2.70[b]
*Leuconostoc mesenteroides*S3	14.10±2.00[d]	12.00±1.24[c]	10.19±1.22[b]	9.11±0.80[c]	4.98±0.36[a]	8.03±1.00[b]
*Leuconostoc mesenteroides*S1	12.00±0.940[c]	9.02±1.17[b]	8.08±2.57[a]	7.00±0.90[ab]	8.11±0.23[d]	9.00±0.45[c]

Table 7. Physiological reactions of selected isolates of lactic acid bacteria

Isolate code	Growth at 15°C	Growth at 25°C	Growth at 45°C	Growth at pH2.0	Growth at pH3.0	Growth at pH4.0	Growth at pH5.0	Growth at pH8.5	Growth at pH9.6	Growth at 4.5% NaCl	Growth at 6.5% NaCl	Growth at 8% NaCl
*Lactobacillus plantarum*MG1	+	+	-	-	+	+	+	-	-	+	+	-
*Lactobacillus plantarum*Yh1	+	+	+	+	+	+	+	+	+	+	+	+
Lactobacillius plantarum MG2	-	+	+	-	-	-	+	+	+	+	+	+
*Lactobacillus plantarum*Yh2	-	+	-	+	+	+	+	+	-	+	+	-
*Leuconostoc mesenteroides*S2	+	+	+	-	-	-	-	-	-	+	+	+
*Leuconostoc mesenteroides*S3	+	+	+	-	-	+	+	+	+	+	+	+
*Leuconostoc mesenteroides*S1	+	+	+	-	-	-	+	+	+	+	+	-

Key: S = isolates from soya bean flour; MG = isolates from malted grain; Yh = isolates from yoghurt; + = positive, - = negative

the outcomes were significantly different (P<0.05), but MG2 and Yh2 were similar in their Antimicrobial activity. All the other test organisms were also found to have significantly different (p<0.05) susceptibility to the isolates. *Pseudomonas aeruginosa* was inhibited most by S3 with about 12.00±1.24mm, while for *Shigella* spp., the antimicrobial activity of *Lactobacillus plantarum*Yh1 was the highest among the seven selected isolates. S2, S1 and Yh1 had the highest zone of inhibition when tested against *Klebsiella* spp, *Bacillus subtilis and Salmonella typhi* respectively.

The isolates from the fermentation of Soya beans were all at least 6mm in clear zone yielded when tested against the selected pathogenic bacteria. The mechanism of inhibition may range from cell wall degradation via synthesis of bacteriocins and acidification of the alimentary canal which prevents the survival of pathogens that require alkaline pH thus preventing them to cause their pathological effects (Girumf *et al.,* 2010).

Empirically, 25^{o}C favored the growth of the LAB isolates more compared to 15^{o}C and 45^{o}C while pH.5 was the optimum hydrogen ion concentration for the growth of the LAB isolates in the table above. However, 4.5% and 6.5% NaCl supported the growth of the selected LAB species better compared to 8% NaCl concentration. pH. 2.0-9.6 was found to be unfavourable for the growth of S2.

Godwin and Zeikus (2004) hinted in their research that LABs respond differently to varoius physicochemical parameters. However, it was reported by [9,19] that strains of the same species of LAB may vary slightly in their required optimum temperature, pH., and salt concentration etc. This may be beneficial in control of the fermentation of such probiotic products with high yield of biomass for maximum benefits in the industrial production line [23].

4. CONCLUSION

In conclusion the present study showed that the isolates grew well and exhibit excellent functional properties which make them a good potential probiotics that can be vehicle by soya beans for further studies.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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