

Full Length Research Paper

Reduction of oligosaccharide content of soybeans by the action of *Lactobacillus plantarum* isolated from fermented cereals

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Most African foods used in weaning are usually fermented cereals which supplies mainly carbohydrate. Unless these are supplemented with other nutrient sources, they may lead to excessive intake of carbohydrate which might cause malnutrition in growing children. Minimum dietary requirements of a child for protein could be met through fortification with legumes like cowpea, peanuts or soybeans but they contain raffinose family of oligosaccharide (RFOs), which are responsible for gas formation, bloating and flatulence in children. This work aims at using *Lactobacillus plantarum* and the enzyme α - and β -galactosidases it produces, to hydrolyse raffinose to simple sugars and hence improve the raffinose-containing weaning foods. Three strains of *L. plantarum* obtained from fermented cereal gruels and identified using both classical and molecular methods were selected after screening for their ability to produce these enzymes in abundance. They were used to ferment different combinations of cereals and legumes. The oligosaccharide profile before and after the various pre-treatment methods and fermentation were determined using gas chromatography with flame ionization detector (GC-FID). Data obtained were analysed using ANOVA at $p < 0.05$. Fermentation for 120 h with *L. plantarum* reduced the total RFO content of soybeans to different levels in the samples. Losses of 30, 28 and 37% in stachyose, raffinose and verbascose, respectively were observed in the fermented raw samples; 72, 58 and 41% reduction in the fermented cooked samples and 76, 68 and 71% reduction in the roasted samples relative to the untreated samples. The use of galactosidase enzymes by *L. plantarum* from fermented cereals reduced the raffinose-oligosaccharide profile with simultaneous increase in reducing sugar levels. Adequate weaning foods can be prepared with such and the problem of bloating, gas production and flatulence can be solved by the action of *L. plantarum* in legume-cereal blends.

Key words: Raffinose-oligosaccharides, soybeans, fermented cereals, *Lactobacillus plantarum*, alpha-galactosidase, weaning foods.

INTRODUCTION

Raffinose is a trisaccharide composed of galactose, fructose and glucose. It can be found in beans, cowpea, pigeon pea, Bambara groundnut, other vegetables, and whole grains. It can also be found in honey and brown sugar. Raffinose can be hydrolyzed to D-galactose and sucrose by the enzyme alpha-galactosidase and beta-galactosidase (α and β -GAL), enzymes not found in the human digestive tract. Alpha-GAL also hydrolyzes other α -galactosides such as stachyose, verbascose, and galactinol, if present. The enzyme does not cleave β -linked galactose, as in lactose (Storey et al., 1998; Townsend and Pitchford, 2012).

The raffinose family of oligosaccharide (RFOs) is alpha-galactosyl derivatives of sucrose, and the most common are the trisaccharide raffinose, the tetrasaccharide stachyose, and the pentasaccharide verbascose. RFOs are almost ubiquitous in the plant kingdom, being found in a large variety of seeds from many different families, and they rank second only to

sucrose in abundance as soluble carbohydrates (Storey et al., 1998; Townsend and Pitchford, 2012). It is a dextrorotatory trisaccharide, occurring in cotton seed and in the molasses of beetroot, composed of d-galactose, d-glucose, and d-fructose and formed by transfer of d-galactose from UDP-d-galactose (http://www.righthealth.com/corp/doingright).

Production of the enzyme α -galactosidase is a desirable quality of *L. plantarum* that could be harnessed for the breakdown of Raffinose-oligosaccharide into simple sugars α -galactosidase enzyme is known to break down raffinose at the α -1, 4 glycosidic bond while β -galactosidase is able to breakdown the chain at the β -1, 6 positions. The production of α and β galactosidase by *L. plantarum* can be employed to break down the raffinose oligosaccharide that is present in soybeans through fermentation. Also, Rodriguez et al. (2009) opined that *L. plantarum* is the commercial starter most frequently used in the fermentation of food products of plant origin

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because of its ability to produce the enzyme α -galactosidase.

L. plantarum belong to a group of bacteria called lactic acid bacteria (LAB). They are a group of bacteria characterized by their ability to synthesize lactic acid. Typical LAB are gram-positive, non sporing, catalase - negative, devoid of cytochromes, anaerobic but aerotolerant cocci or rods that are acid-tolerant and produce lactic as the major end product during fermentation (Olaoye and Onilude, 2009). Their use in fermented foods dates back to ancient times because of their contribution to taste, aroma, flavour and increased shelf life of foods that contain them. They are however regarded as safe in foods because of their use as 'probiotics' in food. Lactic acid bacteria have also been isolated from several indigenous and African fermented foods such as *Ogi*, *Foofoo*, *Gari*, *kefir*, *kumiss*, *Tofu*, *Mawe* and drinks such as *Nunu*, Palm wine, *Agadagidi*, and *Burukutu*, etc (Oyewole and Odunfa, 1998).

L. plantarum has however been named as one of those good, useful and safe bacteria probiotics, as defined in a FAO/WHO (2002) report, as 'live microorganisms which when administered in adequate amounts confer a health benefit on the host'. Probiotics are beneficial bacteria in that they favourably alter the intestinal microflora balance such as reconstruction of normal intestinal microflora after disorders caused by diarrhoea, antibiotic therapy and radiotherapy. *L. plantarum* inhibits the growth of harmful bacteria, promote good digestion, boost immune function and increase resistance to infection (Ammor et al., 2006). It also exercises some therapeutic effect in the gut by its ability to breakdown complex oligosaccharide which causes bloating and gas production (Cummings and MacFarlane, 2007).

L. plantarum has been implicated in the breakdown of oligosaccharide in the colon, breakdown of unused energy substances and stimulation of cell growth by authors like Cummings and MacFarlane, (2007). It has also been noted that the breakdown of ROF is slower in adults and infants but this can be achieved by the consumption of fermented foods that contains *L. plantarum*. Also, humans cannot digest and absorb carbohydrates like starches, fibre and oligosaccharide without the help of bacteria such as *L. plantarum*. *L. plantarum* also helps people with lactose intolerance to overcome the discomfort they experience by the consumption of such sugars. *L. plantarum* ferments such complex sugars and make it available to host cell in assimilable form as sources of useful energy and nutrient (Gibson and Glenn, 2004).

MATERIALS AND METHODS

Oligosaccharide determination in soybean

Preparation of inocula

The LAB isolates namely *L. plantarum* isolated from fermented cereal gruels and identified using classical and molecular methods and labelled LV1, LV2 and LV3 that were used for the various tests were prepared by inoculating a colony from a 24 h- old culture of each *L. plantarum* streaked on a plate into a sterile 9 ml MRS broth. This was incubated for 24 h at 30°C.

Standardization of inocula

The LAB isolates that were used were standardized according to MacFarland standard using BaCl_2 and HCl at the right proportion. The culture supernatant was also brought to the same optical density (OD) of 0.500 and a colony count of 3.1×10^3 cfu/ml, using sterile MRS broth (Olutiola et al., 1993).

Fermentation of soybeans with the isolates of *L. plantarum*

Two batches each of 1 g, 2 g and 3 g each of the raw, cooked and roasted soybeans were weighed in triplicate into screw capped bottles. The first batch was used for the uncured isolates while the second batch was used for the cured isolates. Sterile distilled water (10 ml) was added to it for the sample to become a paste. 1 ml

each of the standardised inocula of cured and uncured *L. plantarum* were added separately to the two batches and allowed to ferment for five days. Samples were taken for reducing sugar and oligosaccharide content determination every 24 h. A control was set up for the samples with additional 1ml of sterile distilled water in the samples without the organisms.

Production of enzymes alpha and beta-galactosidases

The method of Mital et al. (1973) as modified by Hassan and Durr (1974) were used for the determination and assay of both enzymes.

Determination of raffinose, stachyose and verbascose

The method of Beutler (1998) as modified by Black and Bagley (2007) and Townsend and Pitchford (2012) were used. Milled soybean seed, 0.50 g (to pass a 0.5 mm sieve) and fermented samples were weighed accurately into glass test-tubes (18 × 150 mm). Ethanol, 5 ml (95 % v/v) was added to each tube and incubated at 84 to 88°C for 5 min to inactivate endogenous enzymes. The tube contents were quantitatively transferred to a 50 ml volumetric flask and the volume adjusted to the mark with 50 mM sodium acetate buffer (pH 4.5). The samples were allowed to extract over 15 min with occasional swirling. An aliquot (approx. 5 ml) of this slurry was transferred to a glass test tube (16 × 120 mm). Chloroform, 2 ml was added and mixed vigorously on a vortex mixer for 15 s. It was centrifuged at 1 500 g for 10 min to remove most of the lipids from the aqueous phase into the chloroform (lower phase); and the insoluble plant material that concentrated between the phases. The upper (aqueous) phase was analyzed and diluted accordingly.

Methods

The Oligosaccharide profile of the samples was determined by multiplying the absorbance difference of the blank and samples with the final volume of the mixture and molecular weight of the sample assayed.

The concentration of the oligosaccharides was determined by:

$$c = V \times MW \times \Delta A \text{ [g/L]}$$

Where, V = final volume [mL], MW = molecular weight of the substance assayed [g/mol]
= 6300 [$\text{l} \times \text{mol}^{-1} \times \text{cm}^{-1}$]; v = sample volume [mL].

Raffinose:

$$\begin{aligned} c &= 2.62 \times 504.5 \times \Delta A \text{ raffinose [g/L]} \\ &= 6300 \times 1 \times 0.2 \\ &= 1.049 \times \Delta A \text{ raffinose [g/L]} \end{aligned}$$

Verbascope:

$$\begin{aligned} c &= 2.62 \times 624.59 \times \Delta A \text{ verbascope [g/L]} \\ &= 6300 \times 1 \times 0.2 \\ &= 1.299 \times \Delta A \text{ verbascope [g/L]} \end{aligned}$$

Starchyose:

$$\begin{aligned} c &= 2.62 \times 666.574 \times \Delta A \text{ stachyose [g/100g]} \\ &= 6300 \times 1 \times 0.2 \\ &= 1.386 \times \Delta A \text{ stachyose [g/L]} \end{aligned}$$

The method of Beutler (1998) as modified by Black and Bagley (2007) was used. The GC-FID is an instrument that measures the concentration of organic substances. It is frequently used as a detector in gas-chromatography. The operating principle is based on the detection of organic compounds formed during the breakdown of complex substances. It can measure organic substance concentration at very low and high levels Model is (Perkin-Elmer).

Production of reducing sugars during fermentation

The total reducing sugars produced was determined using the method of Bernfeld (1955). The readings obtained through a spectrophotometer were subjected to a standard curve of glucose. The other sugars were calculated from the curve also.

Statistical analysis

The statistical analyses carried out include analysis of variance,

FERMENTED RAW

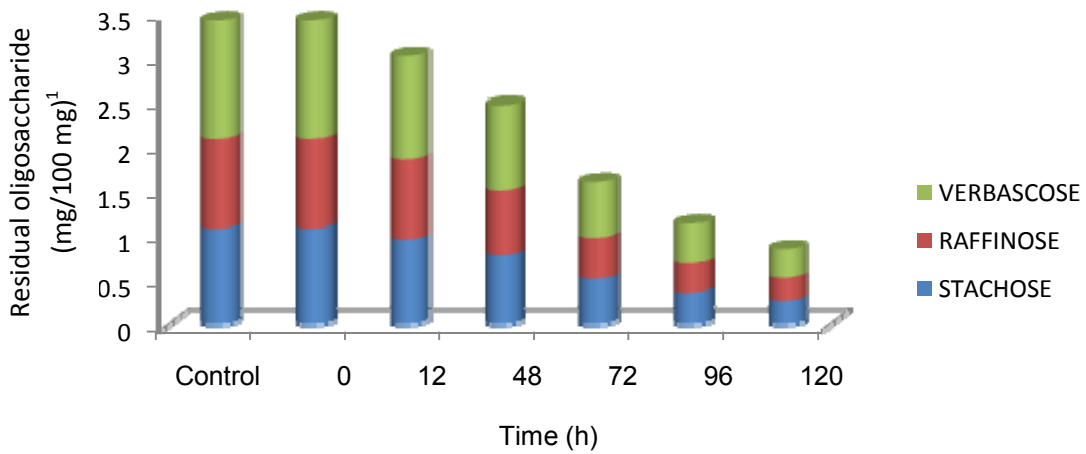


Figure 1. Residual oligosaccharide content of raw soybeans during fermentation with *L. plantarum*. Values are means of replicate determinations shows significance in the samples ($P < 0.05$).

FERMENTED RAW

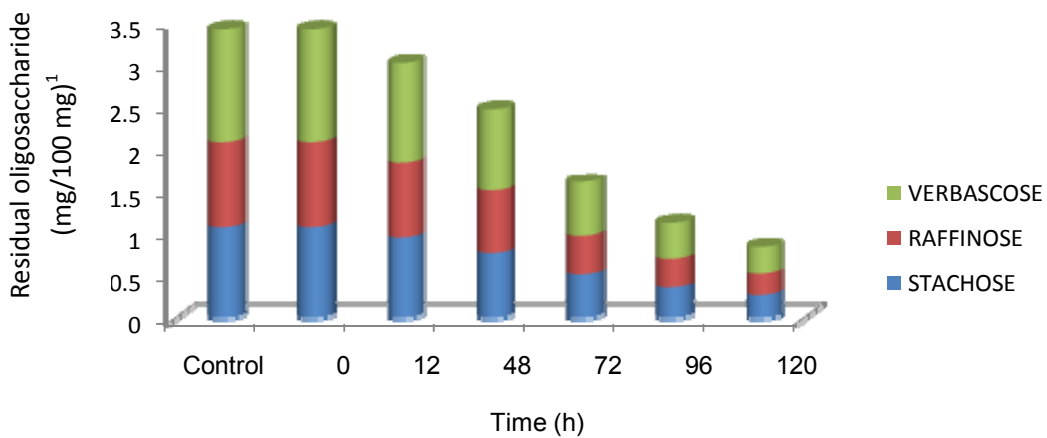


Figure 2. Residual oligosaccharide content of cooked soybeans during fermentation with *L. plantarum*. Values are means of replicate determinations shows significance in the samples ($P < 0.05$).

FERMENTED ROASTED

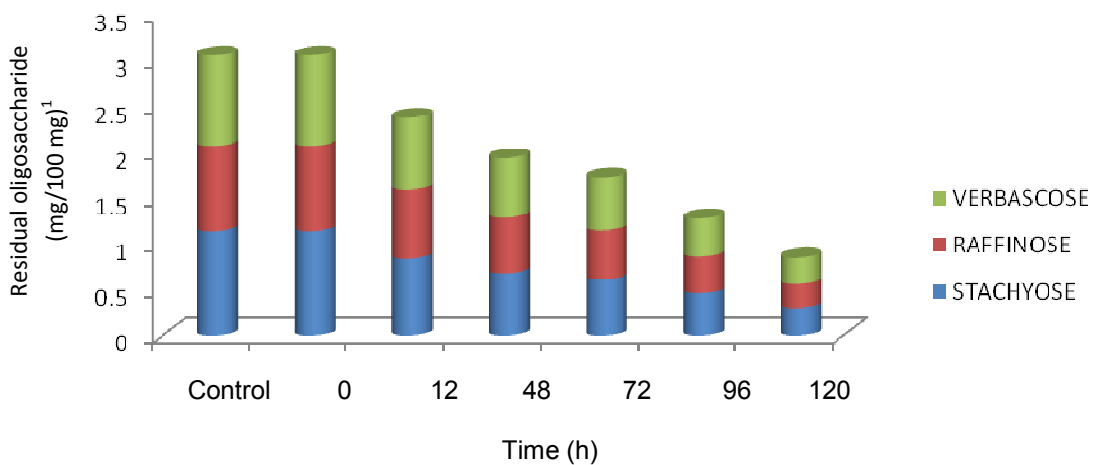


Figure 3. Residual oligosaccharide content of roasted soybeans during fermentation with *L. plantarum*. Values are means of replicate determinations shows significance in the samples ($P < 0.05$).

Duncan multiple range of variables, mean, standard deviation and Standard error using SAS Analytical package.

RESULTS

Figures 1, 2 and 3 show the oligosaccharide content ($\text{mg}/100 \text{ mg}$)¹ of the samples as the fermentation

progresses. *L. plantarum* was able to reduce the oligosaccharide content of the samples, even though the different pre-treatment methods have reduced the oligosaccharide content to an extent, fermentation with *L. plantarum* was able to break it down further, and there was reduction in the oligosaccharide content from 0 to 120 h as observed in the samples.

FERMENTED COOKED REDUCING SUGAR

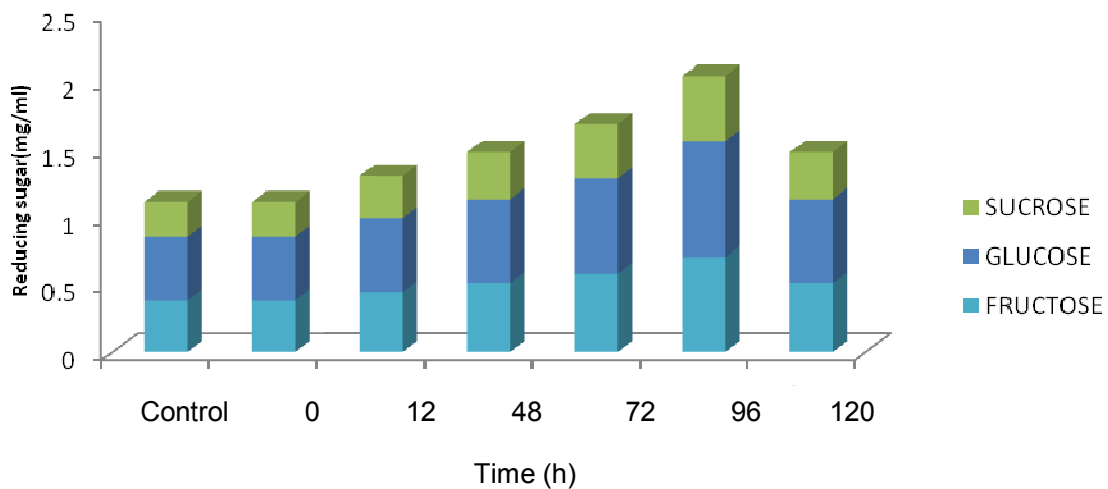


Figure 4. Timed profile of reducing sugar by *L. plantarum* isolate LV1, LV2 and LV3 during fermentation with raw soybeans. Values are means of replicate determinations shows significance in the samples ($P < 0.05$).

FERMENTED COOKED REDUCING SUGAR

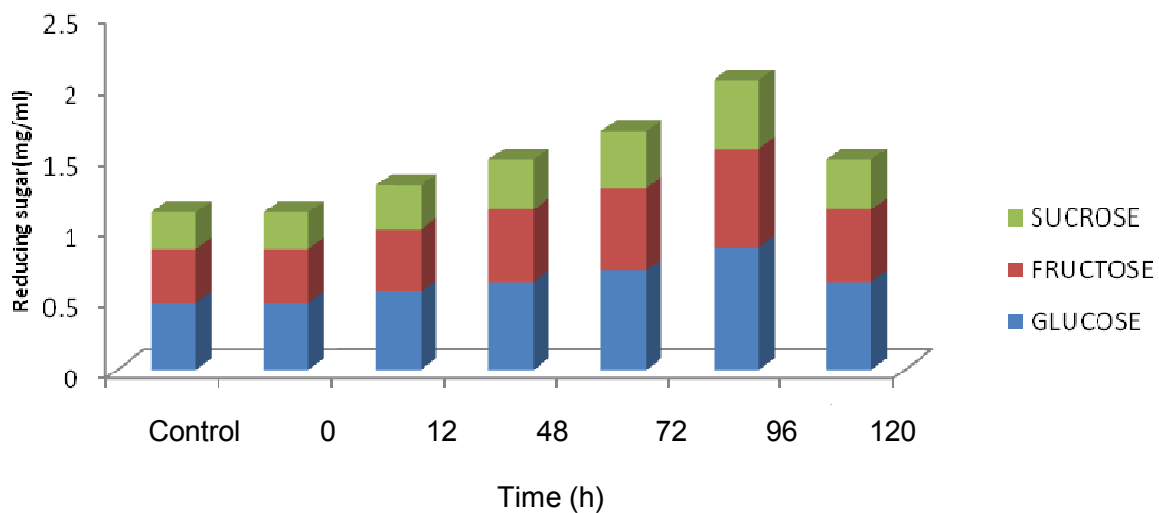


Figure 5. Timed profile of reducing sugar by *L. plantarum* isolate LV1, LV2 and LV3 during fermentation with cooked soybeans. Values are means of replicate determinations shows significance in the samples ($P < 0.05$).

Figure 1 shows the comparative oligosaccharide content ($\text{mg}/100 \text{ mg}$)¹ from cooked soybeans fermentation by *L. plantarum* isolate. Cooking has reduced the oligosaccharide content of the soybeans before fermentation compared to what was found in raw (3.6) to (1.7). With cooked soybeans, there was a reduction in the oligosaccharide content from 0 hr to 120 h. No significant reduction ($p < 0.05$) was observed between 0 to 24 h, but at 48 h of fermentation, there was a reduction from 1.2 to 1.0 at 48 h, 0.9, 0.8 and 0.6 at 72, 96 and 120 h respectively.

Figure 2 shows the comparative oligosaccharide content ($\text{mg}/100 \text{ mg}$)¹ of raw soybeans subjected to fermentation by *L. plantarum*. The oligosaccharide content of the raw sample at 0 h was 3.6, using the isolate; thier reduction was rather slow. No observable change was noticed until the 48th hour (2.6). The oligosaccharide content reduced from 2.0 to 1.7 at 96 h and at 120 h to 1.2.

Figure 3 shows the comparative oligosaccharide content ($\text{mg}/100 \text{ mg}$)¹ of the roasted soybeans subjected to fermentation by *L. plantarum*. Roasting reduced the oligosaccharide content a lot before fermentation from 3.6 in raw to 1.4. The highest oligosaccharide content observed was 1.4, it reduced from 1.4 to 1.1 at 24 h, then to 0.9, 0.8, 0.7 and 0.6 at 48, 96 and 120 h, respectively.

There was a significance reduction ($p = 0.05$) in the oligosaccharide content from 0 -120 h when the organism was used. The pre-treatment method had a lot of significant difference ($p < 0.05$) on the oligosaccharide content as it was possible for fermentation to start immediately at 24 h.

The analysis of variance result shows a significant difference ($p < 0.05$) between the Raw, cooked and roasted soybeans in the reduction of oligosaccharide content, while it was the highest in raw sample (3.6), it was followed by cooked (1.7) and the least was observed in roasted (1.4).

There was a significant difference ($p < 0.05$) in the time interval from 0 - 120 h, also there was a significant difference ($p < 0.05$) in the oligosaccharide content between the *L. plantarum* isolates that was used for fermentation.

Figures 4, 5 and 6 show the effect of fermentation on the reducing sugar (mg/ml) content of the pre-treated soybeans. It could be observed that there was a reduction in the oligosaccharide content from 0 to 96 h when the *L. plantarum* was used for fermentation. The reducing sugar content of the food blend increased from 0 to 96 h when *L. plantarum* was used for fermentation. The oligosaccharides in the samples were broken down by the organism into reducing sugar. At 120 h, the

FERMENTED COOKED REDUCING SUGAR

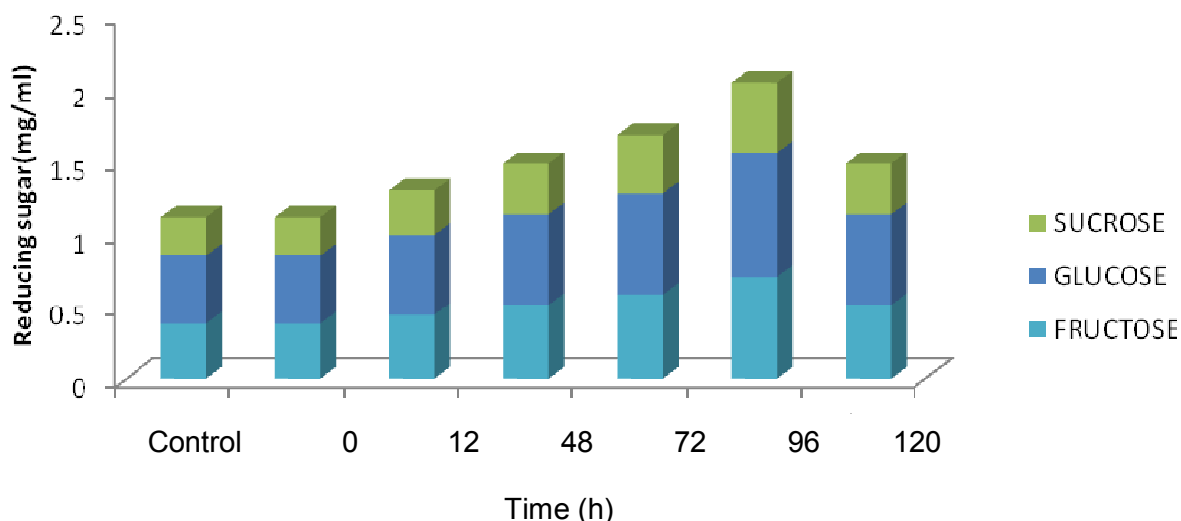


Figure 6. Timed profile of reducing sugar by *L. plantarum* isolate LV1, LV2 and LV3 during fermentation with roasted soybeans. Values are means of replicate determinations shows significance in the samples ($P < 0.05$).

Table 1. Production of α - and β -galactosidases by the selected *L. plantarum*.

Isolate code	Alpha-galactosidase	Beta-galactosidase
<i>L. plantarum</i> Lv1	1.818 \pm 0.002	0.958 \pm 0.005
<i>L. plantarum</i> Lv2	1.820 \pm 0.025	0.947 \pm 0.005
<i>L. plantarum</i> Lv3	1.805 \pm 0.010	0.940 \pm 0.003

reducing sugars reduced again.

Table 1 shows the production of alpha and beta galactosidases by *L. plantarum*. The abundant production of these enzymes was a criterion that was used for selecting the organisms for the fermentation of the samples.

Figure 5 shows a trend in the fermentation pattern when the *L. plantarum* was used to ferment the cooked soybeans sample. There was a significant increase ($p=0.05$) in the reducing sugar production (mg/ml) with the *L. plantarum* sample from 0 to 96 h; there was a drop at 120 h. It increased from 0.58 mg/ml at 0 h to 0.98 mg/ml at 72 h and the peaked at 96 h (1.1 mg/ml) but it reduced to 0.84 mg/ml at 120 h.

Figure 4 shows the reducing sugar (mg/ml) production by *L. plantarum* isolate in the same sample when the raw soybeans was used, there was a gradual increase in the reducing sugar produced from 0 to 96 h being the peak (0.52 mg/ml) and 0 h (0.62 mg/ml) 48 h and (0.96 mg/ml) at 96 h. There was a significant reduction ($p=0.05$) at 120 h to 0.85 mg/ml.

Figure 5 shows the reducing sugar production (mg/ml) by the *L. plantarum* isolate in the roasted sample. There was an increase in the reducing sugar from 0 to 72 h respectively, from 0.62 to 0.72 and 1.0 mg/ml. The peak was observed at 96 h (1.8 mg/ml) and a reduction to 0.98 at 120 h. There was also a significant difference ($p=0.05$) in the various time intervals as significant changes took place in the cooked and roasted samples but this was not observed in the raw samples.

DISCUSSION

The breakdown of raffinose family of oligosaccharide was achieved by the use of *L. plantarum* to ferment the different soybeans sample. Accompanying the breakdown of oligosaccharide is the production of reducing sugars which the complex sugar is broken down into.

The effect of processing on the sugars and total RFO

content of the legume revealed that cooking in water and roasting resulted in a loss of RFO to an extent when compared with the raw samples. The results agree with those earlier reported by Burbano et al. (1990) and Hymowitz (2012) who established that oligosaccharide content of legumes was influenced and reduced by different pre-treatments methods and environmental factors; but fermentation is able to reduce it to a level that is safe for consumption. Both processes involved the use of heat.

Hence, the reductions may have been due to heat induced hydrolysis of the oligosaccharide to simple disaccharides. These findings are in agreement with those reported for cowpeas and other legumes in the work of Oboh et al. (2000). Cooking also resulted in slight loss of sugar which is in agreement with the result of Somlari and Balogh (1993).

Fermentation for 120 h with *L. plantarum* reduced the total RFO content of soybeans to different levels in the samples. Losses of 30, 28 and 37% in stachyose, raffinose and verbascose respectively were observed in the fermented raw samples. A 72, 58 and 41% reduction in stachyose, raffinose and verbascose respectively was observed in the fermented cooked samples. Fermented roasted samples showed a 76, 68 and 71% reduction in stachyose, raffinose and verbascose respectively when compared to raw seeds. These losses were significant at the different fermentation time. This is also similar to the findings of Mulimani and Ramaligam (1995) who reported complete hydrolysis of stachyose and raffinose to more than 60% hydrolysis due to fermentation.

The reduction of RFO in the raw sample was small when compared to the other samples. This may be as a result of the relative hardness of the seed coat which limits the uptake of water and may prevent thorough fermentation of the samples. Significant reduction in the RFO content has been reported during fermentation and other forms of pre-treatment by different authors (Akinyele and Akinlosotu, 1991). The pre-treatment was however necessary to make the samples amenable to LAB

fermentation. This is because it was not easy to ferment the raw samples with the hard seed coat but the pre-treatment methods have solved this problem.

The changes in total RFO content due to fermentation with *L. plantarum* indicated a significant reduction in all the samples due to the metabolic activity of *L. plantarum*. Similar findings have been reported for related leguminous plants such as cowpea, African yam beans, Jack beans and Pigeon peas by Akinyele and Akinlosotu (1991) and Oboh (2000). The relative reduction in the RFO observed in the study could be attributed to the presence of both alpha and beta-degradation of the sugars at alpha 1, 4 and beta - 1, 6 positions, respectively. This is similar to that obtained by Oboh and co-workers (2000). This is because *L. plantarum* produce the enzyme alpha-galactosidase in the medium of growth which is able to breakdown the oligosaccharides of the RFO.

A reduction rate of about 20% was observed in all the sugars at every 24 up to 96 h. This is similar to the findings of Leblanc et al. (2004) who reported that *L. plantarum* was able to eliminate raffinose, a non-digestible alpha-oligosaccharide (NDO) found in soy products. The combination of the various pre-treatment methods and fermentation of soybeans have caused a reduction in the RFO and ANF. This has led to increase in the nutritional composition of the food in which it is added, it also increased the availability of minerals, for example Ca^{2+} and Fe^{2+} . This observation are in agreement with earlier studies by Onilude et al. (1999) and Wakil and Onilude (2009).

There was however, a relative increase in the reducing sugars profile, a 61, 53 and 32% increase was observed in the glucose, fructose and sucrose after the raw samples were fermented with *L. plantarum*. The fermented cooked samples experienced a 78, 64 and 44% increase in glucose, fructose and sucrose, respectively while the fermented roasted samples experienced a percentage increase of 87, 71 and 52, respectively during fermentation of all the samples with *L. plantarum* from 0 to 96 h. Similar findings was reported by Muzquiz et al. (1993) who reported increase in the reducing sugar profile as the total RFO reduced due to its breakdown during fermentation by the enzymatic activities of microorganisms. The reducing sugar in the fermenting soybeans increased from 24 to 96 h and decreased thereafter in all the fermentation procedures. The period of rapid increase coincides with the period of increased total reducing content, reduced RFO, and alpha and beta galactosidase activity. There was a reduction at 120 h. The decrease in reducing sugar level of the fermenting powdered samples indicates that they are being used by *L. plantarum* for metabolism. Similar result was reported by Omafuvbe et al. (2007).

The use of intracellular alpha-galactosidase from *L. plantarum* to breakdown raffinose, stachyose and verbascose in raw, cooked and roasted soybeans was achieved. The optimum conditions for the enzymic hydrolysis of raffinose, stachyose and verbascose was pH 5.5 at 50°C for 24 h to 96 h. Alpha-galactosidase showed optimum activity at pH 5.0 and 50°C, with the substrate p-nitrophenyl-alpha-D-galacto-pyranoside (PNGP). The enzyme activity showed detectable loss in reducing sugar production after 96 h. This is also similar to the findings observed in work of Mulimani and Ramalingam (1995) on enzymic hydrolysis of raffinose and stachyose in soymilk by alpha-galactosidase.

Another important quality of a desired food weaning blend is one with a low raffinose-oligosaccharide content in which the complex sugar has been reduced to a level that is digestible or metabolisable by the infants as well as making the reducing sugar readily available for energy production. This was achieved in the food blend formulated in this work. With the use of *L. plantarum*, there was a reduction in the oligosaccharide content of roasted soybeans from 0 to 96 h. Furthermore, there was

an increase in the reducing sugar content of the soybeans during fermentation. This corroborates the work of Espona and Ruperez (2006) which reported a reduction of galacto- oligosaccharide content of soybeans during fermentation. The reduction of RFO, ANF and other unwanted or toxic substances during fermentation and improvement of the nutritional composition of the food by *L. plantarum* is one of the attributes of a good starter culture. The results of this study agrees with that of Le Blanc et al. (2004) on selection of the optimum growth conditions of *L. plantarum* with elevated levels of alpha-gal to be used in the reduction of non- reducing-oligosaccharide in soy products when used as starter cultures (Amankwah et al., 2012; Nagai et al., 2012).

The presence of trisaccharide- oligosaccharide raffinose, a member of RFO in soybeans also received a good treatment. *L. plantarum* produces enzyme α - and β -galactosidase that are able to break down the foods in which the consumption of raffinose is high (Hymowitz, 2012). Apart from the discomfort that is usually experienced, it results in vomiting, diarrhoea, weight loss and malnutrition, because the little child cannot metabolise the complex sugar if consumed on a regular basis, malnutrition sets in. *L. plantarum* was thus able to metabolise the complex sugar and break it down to simple sugars in a manner that is readily available and metabolisable by the growing infants so that they can use such for growth.

L. plantarum is able to metabolize the large amount of complex sugars that are present in soybeans and therefore reduce or remove the limited use and the barrier to wide use of the leguminous food plant.

Conflict of Interest

The author(s) have not declared any conflict of interests.

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