



# Effect of Fermentation Time of *Bacillus subtilis* ATCC 6051 on Black and Red Lima Bean (*Phaseolus lunatus*) Seeds on Nutrients, Antinutrients and Antioxidant Potential

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## Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

## Article Information

DOI: <https://doi.org/10.9734/bji/2024/v28i6758>

## Open Peer Review History:

This journal follows the Advanced Open Peer Review policy. Identity of the Reviewers, Editor(s) and additional Reviewers, peer review comments, different versions of the manuscript, comments of the editors, etc are available here: <https://www.sdiarticle5.com/review-history/122426>

Original Research Article

Received: 02/08/2024

Accepted: 04/10/2024

Published: 11/12/2024

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## ABSTRACT

**Aims:** The lima bean (*Phaseolus lunatus*) is a species of legume rich in protein and minerals, particularly potassium, phosphorus, calcium and iron. However, this legume is not widely consumed because it is considered a poor man's protein and its consumption leads to digestion problems due to its high content of anti-nutritional factors. The aim of this study is therefore to add value to *Phaseolus lunatus* by improving its nutritional and antioxidant quality through controlled fermentation with *Bacillus subtilis* ATCC 6051 in order to make it a food ingredient.

**Study Design:** *Phaseolus lunatus* seed flour was fermented with *Bacillus subtilis* ATCC 6051 for 24, 48 and 72 hours under controlled conditions.

**Methodology:** The impact of fermentation time with *Bacillus subtilis* on the proximal composition, anti-nutritional factors and antioxidant capacity of lima bean flour was evaluated.

**Results:** Protein and fibre contents increased during fermentation (19.77 to 22.93%) and (5.99 to 8.28%) while lipid and carbohydrate contents decreased. The levels of anti-nutritional factors decreased significantly ( $p < 0.05$ ). Antioxidant power increased significantly ( $p < 0.05$ ) during fermentation.

**Conclusion:** Fermentation with *Bacillus subtilis* improves the nutritional quality and antioxidant power of lima bean flour. Lima bean flour fermented with *Bacillus subtilis* could be used as a human food ingredient.

**Keywords:** Lima bean; fermentation; nutrient; anti-nutrition factors; antioxidant activity; *Bacillus subtilis*.

## 1. INTRODUCTION

The majority of the African population is faced with the problem of undernourishment (Yabile, 2011). The consumption of legumes appears to be an alternative to this problem (FAO, 1988). Among these legumes, *Phaseolus lunatus* (L) is a species of bean belonging to the Fabaceae family. This species of bean is very rich in phytochemicals, proteins and minerals, particularly potassium, phosphorus, calcium and iron. The high protein content of lima beans (*Phaseolus lunatus*) could also compensate for the growing protein deficiency in developing countries due to the high price of animal protein" (Young and Pellett, 1994). However, "this legume is not widely consumed because it is considered a poor man's protein and its consumption poses a problem in terms of digestion and the bioavailability of certain nutrients due to the high content of anti-nutritional factors (Fadahunsi, 2009). Lima bean seeds contain anti-nutritional factors such as hydrogen cyanide, phytic acids, saponin, oxalate, tannin, trypsin-inhibiting activity and agglutinating activity (Ezeagu and Ibegbu, 2010). It has been observed that these anti-nutritional factors inhibit the absorption of nutrients and their subsequent use and assimilation by the animals. In addition, they cause damage to certain organs such as the liver, kidneys and spleen (Emiola et al. 2007). It is therefore important to find ways of solving this problem and to add value to lima bean in order to increase its consumption and production.

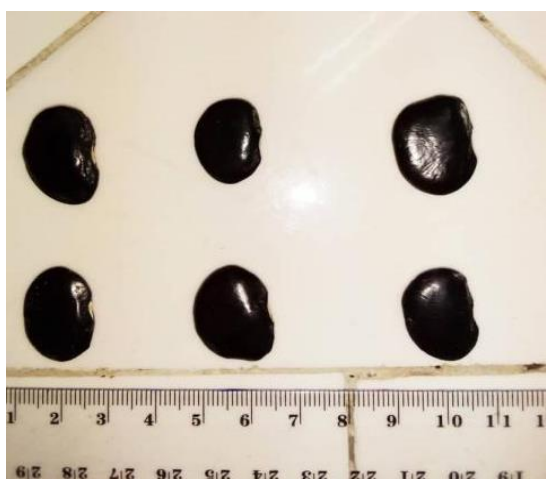
"Fermentation is a desirable process of biochemical modification of the primary food matrix by micro-organisms and their enzymes" (Kahajdova and Karovicova, 2007). Fermentation is used to improve the bioaccessibility and bioavailability of nutrients (Hotz and Gibson, 2007) and to improve organoleptic properties and extend shelf life (Steinkraus, 1994; Chaves-Lopez et al. 2014; Hassan et al. 2014). Food can be fermented naturally or by adding a starter culture. The use of starter cultures is very important in microbiology and plays different roles in a fermented food. Starter cultures improve the safety of fermented foods, either by eliminating spoilage or pathogenic micro-organisms, or by producing bacteriocins that inhibit the growth of undesirable microbes. In addition, by controlling the food microbiota, lactic ferments can extend the shelf life of fermented foods. They also enhance certain organoleptic characteristics of fermented foods, such as colour, aroma, flavour and texture (Laranjo, 2023). Some fermented foods based on legumes indigenous to Asia and Africa have benefited from technological advances thanks to the use of *Bacillus subtilis* strains as lactic ferments. Studies have shown the beneficial impact that using *Bacillus subtilis* for fermentation has had on the nutritional quality of rapeseed (Konkol et al. 2023). In addition, studies have shown that certain traditional Asian foods produced from legumes fermented with *Bacillus subtilis* have various health benefits such as antihypertensive, antidiabetic and anticancer properties (Sanjukta

and Rai, 2016; N'zi et al. 2021). In addition, *Bacillus subtilis* strains are used to produce a range of fermented foods, including sauerkraut, pickles, olives, vinegar, dairy products and others. Thus, the aim of this study is to show the effect of controlled fermentation with *Bacillus subtilis* on the nutritional quality and antioxidant power of lima beans.

## 2. MATERIALS AND METHODS

### 2.1 Sample Collection and Flour Production

The biological material used for this study consisted of mature seeds of the black cultivar with red spots of *Phaseolus lunatus* (L.) (Fig. 1). It was supplied by a grower from the villages of Assoumoukro (M'batto) and N'guessankro (Bongouanou), a park in central-eastern Côte d'Ivoire. Samples of dried bean seeds were cleaned and sorted according to size and the absence of foreign or abnormal odours and live or dead insects. The bean samples were finally ground in an appropriate analytical mill and sieved through a 0.5 mm mesh sieve.



**Fig. 1. Mature seeds of the red spotted black cultivar of *Phaseolus lunatus* (L.)**

### 2.2 Fermentation Experiments

Approximately 200 g of lima bean flour was transferred to 4 flat-bottomed flasks of 500 ml each and autoclaved at 121°C for 15 min. The sterilised lima bean flours were aseptically suspended in sterile distilled water at a concentration of 300 g/L. The suspensions were aseptically inoculated with *Bacillus subtilis* ATCC 6051 isolated from lima beans at a concentration of  $4 \times 10^5$  CFU/ml and incubated at 37°C for 24,

48 and 72 hours. After fermentation, the samples were dried in an oven at 45°C and ground in a Moulinex, then sieved through a 0.25 mm mesh sieve. The flours obtained were then stored in glass jars at room temperature until use (Fadahunsi, 2009). Four samples and three replicates per sample were used in the statistical analysis.

### 2.3 Proximate Analysis

The various samples produced at different fermentation times (0, 24, 48, 72 h) were analysed for ash, crude fat, crude protein and crude fibre (AOAC, 1990) in proportions of 1 g each, using standard methods. Carbohydrates were calculated by difference based on the total composition of fermented and unfermented flours (Muller and Tobin, 1980).

Total sugars were determined according to the technique described by Dubois et al. (1956) using phenol and concentrated sulphuric acid. The ethanol-soluble extract (150 µL) was taken and placed in a test tube. Next, 1 mL phenol (5%, w/v) and 1 mL concentrated sulphuric acid (97%) were added. The reaction medium was homogenised and allowed to cool for 5 min. The optical density was read at 490 nm on a spectrophotometer against a control containing all the products except the ethanol-soluble extract. The optical density was converted to total sugars using a standard curve obtained from a glucose solution (1g/L).

Reducing sugars were determined according to the method of Bernfeld (1955) using 3,5-dinitrosalicylic acid (DNS). A volume of 150 µL of ethanol-soluble extract was taken and placed in a test tube. To this volume, 300 µL of DNS was added. The mixture was heated in a boiling water bath for 5 min. 2 mL of distilled water was added to the reaction medium after cooling for 5 min on the bench. The optical density was read at 540 nm with a spectrophotometer against a control containing all the products except the ethanol-soluble extract. The optical density was converted into the quantity of reducing sugars using a standard curve obtained from a glucose solution (1g/L).

### 2.4 Mineral Content Analysis

The following minerals: magnesium (Mg), calcium (Ca), zinc (Zn), iron (Fe), potassium (K) and sodium (Na) were determined by atomic absorption spectrophotometry as described by the AOAC (1990) method.

## 2.5 Anti-nutritional Factors Estimation

Hydrocyanic acid was analysed [15]. Ten (10) g of flour were homogenised in 200 ml of distilled water. The trapped distillate was left to stand for 3 hours and filtered through Whatman paper. The filtrate obtained was distilled with 20 ml sodium hydroxide (0.1 N) and 2 ml KI (0.02 N). The distillate was titrated with silver nitrate AgNO<sub>3</sub> (0.02 N) until a yellowish haze appeared.

The tannin content was estimated spectrophotometrically by the procedure described by Bainbridge et al. (1996). One millilitre of methanolic extract is introduced into a test tube to which 5 mL of vanillin reagent were added. The tube was left to stand for 20 min in the dark and the absorbance was read with a spectrophotometer at 500 nm against a blank. The blank was prepared for each test by adding 5 mL of distilled water to the test tubes replacing the vanillin reagent. The amount of tannins in the sample was determined using a standard range established from a tannic acid solution (2 mg / mL) under the same conditions as the test.

Phytic acid was determined using the procedure described by Latta and Eskin (1980). One gram of flour was homogenized in 20 mL of HCl (0.65). The mixture obtained is stirred for 12 hours at room temperature. The mixture was centrifuged at 3000 trs / min for 40 minutes. To 0.5 mL of supernatant, 3 mL of Wade's reagent were added. The blank was prepared for each sample with 0.5 mL of distilled water in the test tubes without Wade's reagent. The tubes were left to stand for 20 min in the dark and the optical density was read with a spectrophotometer at 490 nm against a blank. The amount of phytate was determined using a standard range established from a sodium phytate solution (10 mg / mL) under the same conditions as the test.

The oxalate was determined using the method of Day and Underwood (1986). Two grams of flour were homogenized in 75 mL of H<sub>2</sub>SO<sub>4</sub> (3M). The mixture was stirred magnetically for 1 hour at room temperature. The whole was filtered through Whatman filter paper. Twenty-five millilitres of filtrate were titrated hot with a solution of potassium permanganate (KMnO<sub>4</sub>, 0.05 M) until the change to persistent pink.

## 2.6 Assessment of Antioxidant Activity

DPPH free radical scavenging potential was assessed on fermented and unfermented flours

that had undergone hydromethanolic maceration. The capacity to trap 'stable' DPPH free radicals in the crude extract of each of the different flour samples was monitored using the method of Hatano et al. (1988). Approximately 1 mL of a methanolic solution of DPPH (3 mM) was added to 2.5 mL of crude extract at a concentration of 4 mg/mL. The mixture was shaken vigorously and allowed to stand for 30 min in the dark. DPPH reduction was determined by measuring absorbance at 517 nm. A control was carried out by also measuring the absorbance of the DPPH solution (3 mM). The oxidative activity of the extract from each of the samples, reflected by the discolouration of the DPPH solution, was calculated as a percentage of inhibition using the following equation:

$$\text{Inhibition (\%)} = \frac{A_{\text{Control}} - A_{\text{sample}}}{A_{\text{Control}}} \times 100$$

$$\text{Fibres brutes (\%)} = \frac{(M_1 - M_2) \times 100}{M_e}$$

$A_{\text{control}}$  = Absorbance of methanolic DPPH solution (3 mM)

$A_{\text{sample}}$  = Absorbance of DPPH solution reduced by sample extract

## 2.7 Statistical Analysis

Analysis of variance (ANOVA) was used to determine differences between treatments. When a difference was observed, Duncan's 5% multiple range test was performed to separate treatment means. Statistical tests were performed using STATISTICA version 7 software.

## 3. RESULTS

### 3.1 Biochemical Composition

The biochemical composition of Lima bean (*Phaseolus lunatus*) seed flour at different fermentation times controlled by *Bacillus subtilis* is presented in Table 1. Protein content increased significantly ( $p < 0.05$ ) as fermentation time increased, from  $19.77 \pm 0.10\%$  to  $22.93 \pm 0.16\%$  after 72 hours. Unlike protein content, lipid content decreased during fermentation. From 0 to 72 hours of fermentation, it varied from  $1.67 \pm 0.06$  to  $1.19 \pm 0.05$ . Fibre content increased during controlled fermentation of the bean flour. From 0 to 72 hours, it increased from  $5.99 \pm 0.11$  to  $8.28 \pm 0.07\%$ . Furthermore, the ash content of the fermented flour remained constant from 0 to

24 hours with a value of  $4.00 \pm 0.00$  g/100 g. From 24 to 72 hours, a significant decrease ( $p < 0.05$ ) was observed with respective values of  $4.00 \pm 0.00$  and  $3.2 \pm 0.09$  g/100 g.

Carbohydrate content decreased significantly ( $p < 0.05$ ) with increasing fermentation time. It decreased from  $68.57 \pm 0.14\%$  to  $64.41 \pm 0.09\%$ . The total sugar content also decreased with increasing fermentation time. The variation was significant ( $p < 0.05$ ) from 0 to 72 hours, with values of  $4.18 \pm 0.02$  and  $2.16 \pm 0.01$  g/100 g respectively. The reducing sugar content showed a bell-shaped trend with an increase from 0 to 48 hours and a decrease after 48 hours. From 0 to 48 hours, the increase was significant ( $p < 0.05$ ) with values of  $39.45 \pm 0.42$  mg/100 g and  $83.58 \pm 0.45$  mg/100 g respectively, and from 48 to 72 hours, the decrease was also significant ( $p < 0.05$ ) with values of  $83.58 \pm 0.45$  and  $43.51 \pm 0.58$  mg/100 g respectively.

### 3.2 Mineral Composition

The mineral composition of lima bean flour at different controlled fermentation times is presented in Table 2. Before inoculation of the flour with the *Bacillus subtilis* starter, the sodium (Na) concentration was  $15.8 \pm 0.2$  mg/100 g. After inoculation with the *Bacillus subtilis* starter, it increased progressively to reach a value of  $27.9 \pm 0.5$  mg/100 g at 72 hours. This increase was significant between 0 and 48 hours of fermentation and was not significant between 48 and 72 hours. Magnesium (Mg) content decreased throughout fermentation. This decrease was significant ( $p < 0.05$ ) between 0 and 48 hours and was not significant ( $p < 0.05$ ) between 48 and 72 hours. In addition, the P content decreased significantly in the first 24 hours following inoculation with *Bacillus subtilis*, with values ranging from  $514.5 \pm 0.51$  mg/100 g to  $512.5 \pm 0.3$  mg/100 g. It then increased until reaching a value of  $552.3 \pm 0.26$  mg/100 g at 72 hours of fermentation. The K content of *Phaseolus lunatus* flour increased significantly ( $p < 0.05$ ) as a function of the controlled fermentation time. Between 0 and 72 hours of fermentation, the values increased from  $1912.2 \pm 0.1$  mg/100 g to  $2076.8 \pm 0.1$  mg/100 g. As with K, Ca content increased significantly ( $p < 0.05$ ) after inoculation of *Phaseolus lunatus* seed flour with *Bacillus subtilis*. It varied to reach a value of  $148.9 \pm 1$  mg/100 g at 72 hours. Fe content decreased from  $12.9 \pm 0.1$  mg/100 g to  $7.3 \pm 0.4$  mg/100 g. Unlike the other minerals, which

varied during fermentation, Cu and Zn content did not. They remained at  $0.5 \pm 0.1$  mg/100 g and  $2.4 \pm 0.1$  mg/100 g respectively.

### 3.3 Antinutritional Factors

The composition of anti-nutritional factors in lima bean flour at different fermentation times is presented in Table 3. Analysis of the results showed that levels of anti-nutritional factors decreased significantly ( $p < 0.05$ ) with increasing fermentation time. Tannin content decreased over the 72 hours of controlled fermentation. It varied significantly from  $61.65 \pm 0.12$  mg/100 g to  $38.16 \pm 0.73$  mg/100 g. Like tannins, phytates decreased from  $53.50 \pm 0.95$  mg/100 g to  $40.35 \pm 0.50$  mg/100 g. Oxalate content also decreased significantly ( $p < 0.05$ ) during fermentation. French It varied from  $221.83 \pm 3.17$  mg/100 g to  $171.41 \pm 1.59$  mg/100 g. Like all the other anti-nutritional factors, the hydrocyanic acid content decreased throughout the fermentation period. This reduction was significant ( $p < 0.05$ ) with values ranging from  $9.64 \pm 0.02$  mg/100 g to  $6.80 \pm 0.01$  mg/100 g between 0 and 72 hours of fermentation.

### 3.4 Antioxidant Activity

The phenolic extracts of flour in controlled fermentation showed DPPH radical inhibition percentages greater than 50% at a concentration of 4 mg/mL in the extraction solvent. These were lower than for vitamin C, which had a value of  $96.03 \pm 0.17\%$ . The DPPH radical inhibition percentages of phenolic extracts from fermenting flour are shown in Fig. 2. They increased significantly ( $p < 0.05$ ) during fermentation from  $75.68 \pm 1.06\%$  to  $89.63 \pm 0.96\%$  between 0 and 72 hours.

## 4. DISCUSSION

Fermentation is a desirable process of biochemical modification of the primary food matrix caused by micro-organisms and their enzymes (Kahajdova and Karovicova, 2007). Controlled fermentations are used to produce a range of fermented foods. *Bacillus* species such as *Bacillus subtilis* and *Bacillus amyloliquefaciens* are widely used to produce fermented foods from soybean and carob in Asia and West Africa respectively (Kimura and Yokoyama, 2019).

During controlled fermentation of *Phaseolus lunatus* (L) bean flour, protein content increased

**Table 1. Changes in the biochemical composition of lima bean flour during fermentation by *Bacillus subtilis***

Fermentation time (h)	Proteins (%)	fat (%)	Fiber (%)	Ash (%)	Carbohydrates (%)	Total sugar (g/ 100 g)	Sugar reducers (mg/ 100 g)
0	19.77 ± 0.10 <sup>a</sup>	1.67± 0.06 <sup>d</sup>	5.99± 0.11 <sup>a</sup>	4.00 ± 0.00 <sup>c</sup>	68.57± 0.14 <sup>c</sup>	4.18 ± 0.02 <sup>d</sup>	39.45± 0.42 <sup>a</sup>
24	21.90± 0.07 <sup>b</sup>	1.50± 0.03 <sup>c</sup>	7.14± 0.09 <sup>b</sup>	4.00 ± 0.00 <sup>c</sup>	65.46± 0.13 <sup>b</sup>	3.25 ± 0.03 <sup>c</sup>	56.26± 0.26 <sup>c</sup>
48	22.04±0.13 <sup>b</sup>	1.32± 0.06 <sup>b</sup>	7.53± 0.14 <sup>c</sup>	3.67±0.29 <sup>b</sup>	65.44± 0.22 <sup>b</sup>	2.33 ± 0.01 <sup>b</sup>	83.58± 0.45 <sup>d</sup>
72	22.93± 0.16 <sup>c</sup>	1.19± 0.05 <sup>a</sup>	8.28± 0.07 <sup>d</sup>	3.20± 0.09 <sup>a</sup>	64.41± 0.09 <sup>a</sup>	2.16 ± 0.01 <sup>a</sup>	43.51± 0.58 <sup>b</sup>

The values are mean ± standard deviation, n = 3; in the columns, the means with different letters indicate a significant difference (p < 0.05)  
Four samples and three replicates per sample were used in the statistical analysis

**Table 2. Changes in mineral content (mg/100 g) of Lima bean flour during fermentation with *Bacillus subtilis***

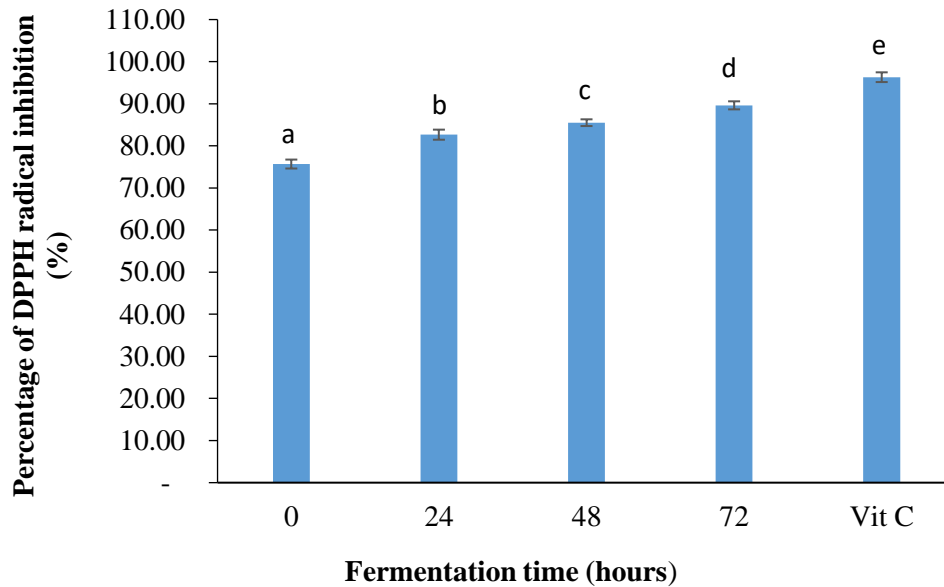
Fermentation time (h)	Mineral content (mg/ 100 g)							
	Mg	P	K	Ca	Na	Fe	Zn	Cu
0	148.0 ± 0.2 <sup>c</sup>	514.5± 0.5 <sup>b</sup>	1912.2 ± 0.1 <sup>a</sup>	103.6± 0.3 <sup>a</sup>	15.8 ± 0.2 <sup>a</sup>	12.9 ± 0.1 <sup>a</sup>	2.4± 0.2 <sup>a</sup>	0.5± 0.1 <sup>a</sup>
24	141.9 ± 0.6 <sup>b</sup>	512.5 ± 0.3 <sup>a</sup>	2013.6± 0.1 <sup>b</sup>	113.7± 0.7 <sup>b</sup>	26.4 ± 0.5 <sup>b</sup>	12.4 ± 0.5 <sup>a</sup>	2.4± 0.1 <sup>a</sup>	0.5± 0.0 <sup>a</sup>
48	140.8 ± 0.1 <sup>a</sup>	530.6 ± 0.7 <sup>c</sup>	2052.3± 0.7 <sup>c</sup>	135.7± 0.5 <sup>c</sup>	27.2 ± 0.3 <sup>c</sup>	9.3 ± 0.4 <sup>c</sup>	2.4± 0.3 <sup>a</sup>	0.5± 0.1 <sup>a</sup>
72	140.6 ± 0.3 <sup>a</sup>	552.3 ± 0.3 <sup>d</sup>	2076.8 ± 0.1 <sup>d</sup>	148.9± 1.0 <sup>d</sup>	27.9 ± 0.5 <sup>c</sup>	7.3 ± 0.4 <sup>d</sup>	2.4± 0.1 <sup>a</sup>	0.5± 0.1 <sup>a</sup>

The values are mean ± standard deviation, n = 3; in the columns, the means with different letters indicate a significant difference (p < 0.05)  
Four samples and three replicates per sample were used in the statistical analysis

**Table 3. Changes in the content (mg/100g) of anti-nutritional factors in bean seed flour (*Phaseolus lunatus*) during fermentation by *Bacillus subtilis***

Fermentation time (h)	Antinutritional factor content (mg / 100 g)			
	Phytates	Oxalates	Tannins	Hydrogen cyanide
0	53.50 ± 0.95 <sup>c</sup>	221.83 ± 3.17 <sup>d</sup>	61.65 ± 0.12 <sup>d</sup>	9.64 ± 0.02 <sup>d</sup>
24	50.88 ± 0.60 <sup>b</sup>	211.75 ± 2.75 <sup>c</sup>	52.85 ± 0.70 <sup>c</sup>	8.52 ± 0.03 <sup>c</sup>
48	41.55 ± 1.09 <sup>a</sup>	188.83 ± 3.17 <sup>b</sup>	45.98 ± 0.63 <sup>b</sup>	7.68 ± 0.20 <sup>b</sup>
72	40.35 ± 0.50 <sup>a</sup>	171.41 ± 1.59 <sup>a</sup>	38.16 ± 0.73 <sup>a</sup>	6.80 ± 0.01 <sup>a</sup>

Value = Mean ± standard deviation, of n=3; in columns, the means assigned different letters (a, b, c, d) indicate a significant difference at the 5% threshold (p < 0.05)  
Four samples and three replicates per sample were used in the statistical analysis



**Fig. 2. Percentage inhibition of the DPPH radical in phenolic extracts of bean (*Phaseolus lunatus*) seed flour during controlled fermentation**

significantly ( $p < 0.05$ ). The increase in the protein content of *Bacillus subtilis* fermented bean flour could be attributed to the possible secretion of certain extracellular enzymes (proteins) such as amylases, linamarase and cellulose (Raimbault, 1998) into the bean substrate by the organisms during fermentation with the aim of using the bean starch as a carbon source (Obboh, 2006). Furthermore, the increased growth and proliferation of the microbial biomass could also be responsible for the increase in protein content in the bean substrate (Hu et al. 2010). Balogun et al. (2017) also observed an increase in protein content in *Prosopis africana* seeds fermented with *Bacillus subtilis* during their work on the comparative study of the physico-chemical analysis of *Prosopis africana* seeds fermented with different starter cultures. Ruan et al. (2020) also obtained similar results, and during the fermentation of soybeans, they noted that their protein content increased with the duration of fermentation. Zhu et al. (2008) also observed an increase in protein content during the fermentation of black soybeans after 24 hours. However, Slowik-Borowiec et al. (2022) observed different results in their study. Their study showed a progressive decrease in the protein content of certain legumes such as peas, mung beans, lentils, soybeans and sunflowers over the course of the fermentation time using *Bacillus subtilis*. Adesanya et al. (2021) also observed a decrease in protein content in locust

bean seeds fermented with *Bacillus subtilis* as fermentation time increased. They associate this drop in protein content with the high proteolytic activities of *Bacillus subtilis*, which break down more proteins into amino acids and ammonia as fermentation increases.

In addition, the lipid content of *Phaseolus lunatus* L. flour dropped significantly ( $p < 0.05$ ) after inoculation with the *Bacillus subtilis* starter. This result is consistent with that of Hu et al. (2010) who studied the characterisation of natto produced from black soybeans fermented with *Bacillus subtilis* (natto) during fermentation. The drop in lipid content could be the result of lipid hydrolysis by lipases produced by *Bacillus subtilis* strains, which hydrolyse triglycerides into fatty acids and sterols during the fermentation process. Adegbehingbe et al. (2014) also observed an increase in the lipid content of cooked *Phaseolus lunatus* L. seeds fermented with *Bacillus subtilis* and *Bacillus pumilus*.

Ash levels decreased significantly ( $p < 0.05$ ) in *Phaseolus lunatus* (L.) seed flours during fermentation controlled by *Bacillus subtilis*. This reduction could be due to the use of mineral salts by the microbial strains for their metabolism (Reeb et al. 2000). This result is consistent with that of Hu et al. (2010) who observed a reduction in the ash content of black soya during fermentation by *Bacillus natto*. Ojewumi et al.

(2018) also obtained similar results. They observed a decrease in ash content during the fermentation of African locust beans by *Bacillus subtilis*. However, it differs from that of Adegbehingbe et al. (2014) who observed an increase in the ash content of cooked *Phaseolus lunatus* (L.) seeds fermented with *Bacillus subtilis*. Jeff-Agboola and Oguntuase (2006) also observed an increase in the ash content of soybeans fermented with *Bacillus sphaericus* for iru production. They attributed the increase in ash content to microbial destruction of anti-nutritional factors, which would be responsible for the increase in certain minerals.

The carbohydrate content of bean flour inoculated with *Bacillus subtilis* decreased during the fermentation process. This reduction in carbohydrate content could be due to the use of carbohydrates as energy sources by the microorganisms. In fact, the microorganisms could produce enzymes (amylases and maltase) to hydrolyse complex sugars (starch) into simple sugars (glucose) that can be easily used by the microorganisms as sources of energy. In addition, *Bacillus subtilis* has significant amylase activity. This high amylase activity could be the cause of this reduction in carbohydrate content. This result is in agreement with that of Adegbehingbe et al. (2014) who studied the effect of controlled fermentation by *Bacillus subtilis* and *Bacillus pumilus* on the nutrient and antinutrient content of cooked bean seeds (*Phaseolus lunatus* L.). In their study, it was observed that the carbohydrate content of *Phaseolus lunatus* (L.) seeds fermented with *Bacillus subtilis* decreased until the fifth day of fermentation. Ojewumi et al. (2018) also observed a decrease in the carbohydrate content of African locust bean during fermentation using *Bacillus subtilis* under aerobic and anaerobic conditions. They associated the reduction in carbohydrate content with the use of some of the sugars by the fermenting organisms for growth and metabolism.

Dietary fibre is largely made up of celluloses and hemicelluloses. During flour fermentation, a progressive and significant ( $p < 0.05$ ) increase in fibre content was observed. This increase in fibre content could be due to the action of enzymes produced by *Bacillus subtilis* to produce easily usable energy sources. These enzymes then break down the other macronutrients to produce these readily usable energy sources, thus increasing the percentage of fibre as fermentation time increases. Chu et al. (2019)

also observed an increase in the fibre content of fermented millet bran compared to unfermented bran during their work on improving the physicochemical and functional properties of dietary fibre from millet bran fermented with *Bacillus natto*. As for Ojewumi et al. (2018), they observed an increase in the percentage of crude fibre during the first 48 hours in two processes (aerobic and anaerobic) for fermenting African locust beans. The percentage of crude fibre then decreased. This evolutionary trend in fibre content was also observed by Omafuvbe et al. (2002). They reported that fermentation increased the percentage of crude fibre by 30% during the first 48 hours and then reduced it by about the same amount during the last 24 hours.

In addition, the total sugar content decreased significantly ( $p < 0.05$ ) during fermentation. This reduction is similar to that observed in *Prosopis africana* seeds fermented with a *Bacillus subtilis* starter according to the work of Balogun et al. (2017) on the comparative study of the physicochemical analysis of *Prosopis africana* seeds fermented with different starter cultures. The reduction in total sugars suggests their use as an energy source by *Bacillus subtilis*. There could also be a reduction in flatulent oligosaccharides such as stachyose, verbascose and raffinose during the controlled fermentation process. Flatulent oligosaccharides are responsible for intestinal gas after consumption of the beans. The content of reducing sugars increases significantly up to 48 hours after inoculation with *Bacillus subtilis* and then decreases. This change in reducing sugar content is consistent with that reported by Ogunshe et al. (2007).

They observed an increase in soluble reducing sugars during the first two days of fermentation (2.0-11.0 mg g<sup>-1</sup>), followed by a decrease during the controlled fermentation of *Prosopis africana* for the production of Afiyo, a traditional African condiment. The increase in reducing sugars could be due to the action of enzymes produced by micro-organisms to produce simple sugars (glucose, fructose). The decrease in content after 48 hours would be due to the use of the reducing sugars produced as sources of energy.

Minerals are essential for the body to function properly. During fermentation of the beans, an increase in sodium content was observed. This result is similar to that of Atere et al. (2020) who observed an increase in sodium content during



fermentation of *Parkia biglobosa* seeds for iru production. Although the sodium content was increased during fermentation, this does not make bean flour a good source of sodium, as the sodium intake for adults is 1,500 mg/day (Karppanen et al. 2005; Strazzullo and Leclercq, 2014). The reduction in magnesium levels is thought to be due to the use of magnesium by micro-organisms for their metabolism. In addition, during fermentation, the reduction in anti-nutritional factors leads to an increase in magnesium bioavailability. Although the magnesium content has been reduced, fermentation makes *Phaseolus lunatus* a good source of magnesium, with a daily intake of 400 mg/day (Costello et al. 2016). Phosphorus is an essential macromineral involved in the structure of bones, teeth, DNA, RNA and the plasma membrane of cells.

During fermentation, an increase was observed after 48 hours. Atere et al. (2020) also observed an increase in phosphorus content during fermentation of *Parkia biglobosa* seeds. Fermentation is therefore beneficial for the phosphorus intake of *Phaseolus lunatus*, whose requirements are estimated at 700 mg/day for adults (Calvo and Lamberg-Allardt, 2015). Potassium is an essential macromineral and electrolyte that plays an essential role in muscle contraction, nerve innervation, blood pH balance and water balance as the most abundant intracellular cation (Yin et al. 2015). During fermentation of *Phaseolus lunatus* (L.) flour by *Bacillus subtilis*, an increase in potassium content was observed. This result is similar to that of Atere et al. (2020), who also observed an increase in the potassium content of *Parkia biglobosa* seeds during fermentation. *Bacillus subtilis*-fermented flour from *Phaseolus lunatus* (L.) is a good source of potassium, with a daily intake of 4.7 mg for adults (Karppanen et al. 2005).

Calcium is an essential macro-mineral that is responsible for many structural components such as bones and teeth, as well as the body's physiological mechanisms (Kiela and Ghishan, 2016). Calcium content increases during fermentation. In addition, the significant reduction in anti-nutritional factors could lead to an improvement in bioavailability, thereby increasing the calcium intake of *Phaseolus lunatus* (L.). Iron is an essential trace element that plays a vital role in oxygen transport and energy metabolism (Kiela and Ghishan, 2016). During fermentation of the beans, there was a reduction in iron

content that was only significant after 48 hours of fermentation. This reduction could be due to the use of iron in the metabolism of the micro-organisms. Furthermore, this reduction in iron content during fermentation had no significant influence on the iron intake of *Phaseolus lunatus* (L.). In fact, the acceptable daily intake is 8 to 18 mg/day for adults (Finnamore et al. 2014) and the reduction in anti-nutritional factors could lead to an increase in iron bioaccessibility.

Zinc is an essential trace element that functions structurally in proteins and catalytically as a component of over 300 different enzymes (Huang et al. 2015). During fermentation, the zinc content of the beans remained constant. The daily zinc intake is 10 mg. *Phaseolus lunatus* (L.) seeds are not good sources of zinc and fermentation does not affect their zinc content. Copper is an essential trace element that acts as a component of many proteins, including many important enzymes (Ma and Bett, 2000). Like zinc, copper levels did not change during fermentation. Fermented *Phaseolus lunatus* (L.) flour could be a good source of copper, with a daily intake for an adult estimated at 1 mg (Livingstone, 2017).

Tannins are plant substances in the polyphenol family that have the ability to precipitate macromolecules such as proteins and carbohydrates, reducing their bioavailability in a food (Smeriglio et al. 2016). They also reduce the nutritional value of a food by preventing protein digestion. During the fermentation period, a significant reduction in tannin content has been observed. Sharma et al. (2015) also observed a decrease in the tannin content of soya fermented with *Bacillus subtilis* for the production of kinema during their work on optimising the transformation of soya into kinema, an alkaline food fermented by *Bacillus*, in relation to a minimum level of anti-nutrients. The reduction in tannin content could be explained by the fact that *Bacillus subtilis* has the ability to secrete tannases, which are tannin hydrolysis enzymes. The hydrolysis of tannins leads to a reduction in their content in the substrate. The significant reduction ( $p < 0.05$ ) in tannins during controlled fermentation helps to improve the nutritional quality of *Phaseolus lunatus* (L.) flour by increasing protein bioavailability.

In the body, the phytate molecule is capable of forming insoluble complexes with essential divalent cations such as Fe<sup>2+</sup>, Zn<sup>2+</sup>, Mg<sup>2+</sup> and

Ca<sup>2+</sup>, etc., thus preventing their bioavailability. In addition, the phytate molecule has the ability to form complexes with proteins and starch, inhibiting their digestion (Nissar et al. 2017) [53]. Their high presence in a food significantly affects its nutritional quality. The phytic acid content of *Phaseolus lunatus* (L.) flour fell significantly during the fermentation period. Yasar and Tosun (2019) observed a reduction in phytate content in soybeans fermented with *Bacillus subtilis* ATCC PTA-6737. This reduction suggests that *Bacillus subtilis* has the capacity to secrete phytase. Phytase is an enzyme that breaks down phytate by dephosphorylating it (Hirvonen et al. 2019).

Dietary oxalate is of plant origin and can be a component of vegetables, nuts, fruit and cereals. In normal individuals, about half of urinary oxalate comes from the diet and the other half from endogenous synthesis. Eating oxalate-rich foods can cause kidney stones (Mitchell et al. 2019). In addition, a diet rich in oxalates can cause hypocalcaemia. Serum calcium regulates the synthesis of parathyroid hormone, which in turn increases tubular reabsorption of calcium and increases bone resorption (Schenck et al. 2006; Rahman et al. 2012). It therefore appears useful to reduce its content in food by treatment. After inoculation of bean meal with *Bacillus subtilis*, the oxalate content of the meal gradually decreased with the duration of inoculation. This result is similar to that of Adegbehingbe et al. (2014), who observed a reduction in the oxalate content of cooked bean meal fermented with *Bacillus subtilis* compared with unfermented cooked bean meal. The reduction in oxalate levels could be due to the activity of enzymes produced by micro-organisms.

Cyanogenic glycosides can be chemically defined as  $\alpha$ -hydroxynitriles and belong to the secondary metabolites of plants (natural products). They are plant constituents derived from amino acids, present in more than 2,500 plant species. Hydrogen cyanide (HCN), an extremely toxic molecule, is produced from the hydrolysis of cyanogenic glycosides (Vetter, 2000). It stops the oxidation of protoplasm in tissue cells and causes dizziness, headaches, loss of consciousness and convulsions with paralysis of the respiratory centre of the brain. The lethal dose of hydrocyanic acid for an adult male is 50 to 60 mg/kg body weight (Codex alimentarius, 1988). It is necessary to reduce the level of hydrocyanic acid in plants before use, and this requires treatment. During flour

fermentation, a significant reduction ( $p < 0.05$ ) in hydrocyanic acid content was observed. This result is similar to that of Adegbehingbe et al. (2014), who observed a reduction in hydrocyanic acid content in cooked bean seeds fermented with *Bacillus subtilis* compared with unfermented cooked seeds.

An increase in the potential for inhibition of the DPPH radical was observed when *P. lunatus* was fermented with *Bacillus subtilis*. This increase in antioxidant activity could be due to the bioconversion of conjugated forms of phenolic acids into simple forms and the improvement in the bioavailability of phenolic compounds by the action of enzymes produced by *Bacillus subtilis*. This increase in antioxidant activity could also be due to the production of bioactive peptides following protein hydrolysis by proteases released by *Bacillus subtilis*. Ali et al. (2018) reported that the DPPH radical inhibition potential of four soybean cultivars fermented with *Bacillus subtilis* for the production of cheonggjang was greater than that of unfermented soybeans. Dajanta et al. (2011) also reported that soybeans naturally fermented with *Bacillus subtilis* for the production of thua nao had higher DPPH radical inhibition potency than unfermented soybeans.

## 5. CONCLUSION

*Bacillus subtilis* is a species of micro-organism used in the production of certain Asian and African condiments. The use of *Bacillus subtilis* in the controlled fermentation of broad beans (*Phaseolus lunatus*) has improved their nutritional quality. Anti-nutritional factors such as phytates, tannins, oxalate and hydrocyanic acid were considerably reduced, thereby increasing the bioaccessibility of nutrients. In addition, the levels of nutrients such as protein, fibre, reducing sugars, calcium, sodium, potassium and phosphorus have increased. The levels of carbohydrates, lipids, ash, total sugars, magnesium and iron have decreased. The increase in protein content makes fermented beans a good source of protein, which could make *Phaseolus lunatus* (L.) flour fermented with *Bacillus subtilis* an ingredient whose consumption would reduce the problems of protein-energy malnutrition and increase its appeal. In addition, fermentation of the flour with *Bacillus subtilis* increases the antioxidant potential by increasing the scavenging potential of the DPPH radical. Fermentation with *Bacillus subtilis* therefore increases the biological quality of the beans.

## DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of this manuscript.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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