Original Research Article

Effect of fermentation time of *Bacillus subtilis*ATCC 6051 on black and red Lima bean (*PhaseolusLunatus*) seeds on nutrients, antinutrients, and antioxidant potential.

ABSTRACT

Aims: The lima bean (*Phaseoluslunatus*) is a species of legume that is rich in protein, but not widely consumed because its consumption leads to digestion problems due to the high content of anti-nutritional factors. The aim of this study is to enhance the value of this legume by improving its nutritional and antioxidant quality through controlled fermentation with *Bacillus subtilis*ATCC 6051.
Study Design: *Phaseoluslunatus* 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, bioactive phenolic compounds 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 ($p < 0.05$). The levels of bioactive phenolic compounds and antioxidant power increased significantly ($p < 0.05$) during fermentation. A strong correlation was observed between changes in the levels of bioactive compounds and antioxidant potential.
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 sutilis*

1. INTRODUCTION

The majority of the African population is faced with the problem of undernourishment [1]. The consumption of legumes appears to be an alternative to this problem [2]. Among these legumes, *Phaseoluslunatus* (L) is a species of bean belonging to the Fabaceae family. This species of bean is very rich in phytochemicals and proteins. The high protein content of lima beans (*Phaseoluslunatus*) could also compensate for the growing protein deficiency in Africa due to the high price of animal protein [3]. However, consumption of these legumes poses a problem in terms of digestion and the bioavailability of certain nutrients due to the high content of anti-nutritional factors [4]. Lima bean seeds contain anti-nutritional factors such as hydrogen cyanide, phytic acids, saponin, oxalate, tannin, trypsin-inhibiting activity and agglutinating activity [5]. 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 [6]. It is therefore important to find ways of solving this problem.

Fermentation is a desirable process of biochemical modification of the primary food matrix by micro-organisms and their enzymes [7]. Fermentation is used to improve the

bioaccessibility and bioavailability of nutrients [8] and to improve organoleptic properties and extend shelf life [9, 10, 11]. 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 [12]. 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 [13]. 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 [14]. 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 *Phaseoluslunatus* (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.

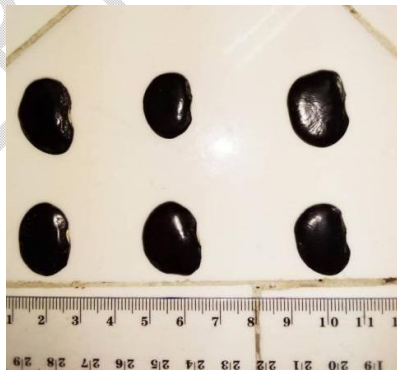

Fig. 1.Mature seeds of the red spotted black cultivar of *Phaseoluslunatus* (L.)

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 $4x10^5$ 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 [4].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 [15] in proportions of 1 g each, using standard methods. Carbohydrates were calculated by difference based on the total composition of fermented and unfermented flours [16].

Total sugars were determined according to the technique described by Dubois et al. 1956 [17] 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 [18] using 3,5-dinitrosalycilic 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 method [15].

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 AgNO3 (0.02 N) until a yellowish haze appeared.

The tannin content was estimated spectrophotometrically by the procedure described by [19]. 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 byLatta and Eskin[20]. 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[21]. Two grams of flour were homogenized in 75 mL of H_2SO_4 (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 ($KMnO_4$, 0.05 M) until the change to persistent pink.

2.6. Determination of bioactive phenolic compounds

Total polyphenols in lima bean flour (fermented and unfermented) were measured using the method described by Singleton et al. [22]. One millilitre (1 mL) of methanolic extract was introduced into a test tube. To the contents of the tube, 1 mL of Folin-ciocalteu reagent was added. The mixture was left to stand for 3 min, then 1 mL of 20% (v/v) sodium carbonate solution was added. The contents of the tube were made up to 10 mL with distilled water and placed in the dark for 30 min. The absorbance was read at 725 nm. The blank was prepared for each test tube by replacing the Folin-ciocalteu reagent with 70% (v/v) methanol. A standard range was established using a stock solution of gallic acid (1 mg/mL) under the same conditions as the test and was used to determine the quantity of phenols in the sample.

Flavonoids were determined using the method described by Meda et al. [23]. A volume of 0.5 mL of methanolic extract was introduced into a test tube. To the contents were successively added 0.5 mL distilled water, 0.5 mL aluminium chloride (10% w/v), 0.5 mL sodium acetate (1 M) and 2 mL distilled water. The blank was prepared for each sample by adding 0.5 mL of distilled water to the test tubes to replace the methanolic extract. The tubes were left to stand for 20 min in the dark and the absorbance was read using a spectrophotometer at 415 nm against a blank. A standard range was established using a quercetin solution (0.1 mg/mL) under the same conditions as the assay.

2.7. 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. [24]. 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:

$$\textbf{Inhibition}\ (\%) = \frac{\mathbf{A_{Control}} - \mathbf{A_{sample}}}{\mathbf{A_{Control}}} \mathbf{x100}$$

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

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

2.8. 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 (*Phaseoluslunatus*) seed flour at different fermentation times controlled by *Bacillus subtilis* is presented in Table 1. Protein content increased significantly ($p < 0.05$) during fermentation from 19.77 ± 0.10% to 22.93 ± 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 ± 0.06 to 1.19 ± 0.05. Fibre content increased during controlled fermentation of the bean flour. From 0 to 72 hours, it increased from 5.99 ± 0.11 to 8.28 ± 0.07%. Furthermore, the ash content of the fermented flour remained constant from 0 to 24 hours with a value of 4.00 ± 0.00 g/100 g. From 24 to 72 hours, a significant decrease ($p < 0.05$) was observed with respective values of 4.00 ± 0.00 and 3.2 ± 0.09 g/100 g.

Carbohydrate content decreased significantly ($p < 0.05$) during fermentation. It decreased from 68.57 ± 0.14% to 64.41 ± 0.09%. The total sugar content decreased during controlled fermentation. The variation was significant ($p < 0.05$) from 0 to 72 hours, with values of 4.18 ± 0.02 and 2.16 ± 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 ± 0.42 mg/100 g and 83.58 ± 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 ± 0.45 and 43.51 ± 0.58 mg/100 g respectively.

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/ 100g)	Sugar reducers (mg/ 100g)
0	19.77 ± 0.10^{a}	1.67± 0.06 ^{d}	5.99± 0.11 ^{a}	4.00 ± 0.00 ^{c}	68.57± 0.14 ^{c}	4.18 ± 0.02^{d}	39.45± 0.42 ^{a}
24	21.90± 0.07^{b}	1.50± 0.03 ^{c}	7.14± 0.09 ^{b}	4.00 ± 0.00 ^{c}	65.46± 0.13^{b}	3.25 ± 0.03^{c}	56.26± 0.26 ^{c}
48	22.04±0.13^{b}	1.32± 0.06 ^{b}	7.53± 0.14 ^{c}	3.67±0.29 ^{b}	65.44± 0.22 ^{b}	2.33 ± 0.01^{b}	83.58± 0.45 ^{d}
72	22.93± 0.16^{c}	1.19± 0.05 ^{a}	8.28± 0.07 ^{d}	3.20± 0.09 ^{a}	64.41± 0.09 ^{a}	2.16 ± 0.01^{a}	43.51± 0.58 ^{b}

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.

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 ± 0.2 mg/100 g. After inoculation with the *Bacillus subtilis* starter, it increased progressively to reach a value of 27.9 ± 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 ± 0.51 mg/100 g to 512.5 ± 0.3 mg/100 g. It then increased until reaching a value of 552.3 ± 0.26 mg/100 g at 72 hours of fermentation. The K content of *Phaseoluslunatus* 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 ± 0.1 mg/100 g to 2076.8 ± 0.1 mg/100 g. As with K, Ca content increased significantly ($p < 0.05$) after inoculation of *Phaseoluslunatus* seed flour with *Bacillus subtilis*. It varied to reach a value of 148.9 ± 1 mg/100 g at 72 hours. Fe content decreased from 12.9 ± 0.1 mg/100 g to 7.3 ± 0.4 mg/100 g. Unlike the other minerals, which varied during fermentation, Cu and Zn content did not. They remained at 0.5 ± 0.1 mg/100 g and 2.4 ± 0.1 mg/100 g respectively.

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

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

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

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 the levels of anti-nutritional factors decreased significantly ($p < 0.05$) during controlled fermentation. Tannin content decreased over the 72 hours of controlled fermentation. It varied significantly from 61.65 ± 0.12 mg/100 g to 38.16 ± 0.73 mg/100 g. Like tannins, phytates decreased from 53.50 ± 0.95 mg/100 g to 40.35 ± 0.50 mg/100 g. Oxalate content also decreased significantly ($p < 0.05$) during fermentation. French It varied from 221.83 ± 3.17 mg/100 g to 171.41 ± 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 ± 0.02 mg/100 g to 6.80 ± 0.01 mg/100 g between 0 and 72 hours of fermentation.

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

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

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.

3.4.Bioactive phenolic compounds

Changes in polyphenol and flavonoid content during fermentation are shown in Figs. 2 and 3 respectively. Analysis of the results showed significant variations ($p < 0.05$) over the course of their development. The polyphenol content increased during fermentation, with values ranging from 199.41 ± 0.91 mg/100 g to 328.37 ± 1.98 mg/100 g between 0 h and 72 h. Total flavonoid content also increased, as did total polyphenol content. It varied from 4.27 ± 0.07 mg/100 g to 5.89 ± 0.06 mg/100 g between 0 h and 72 h.

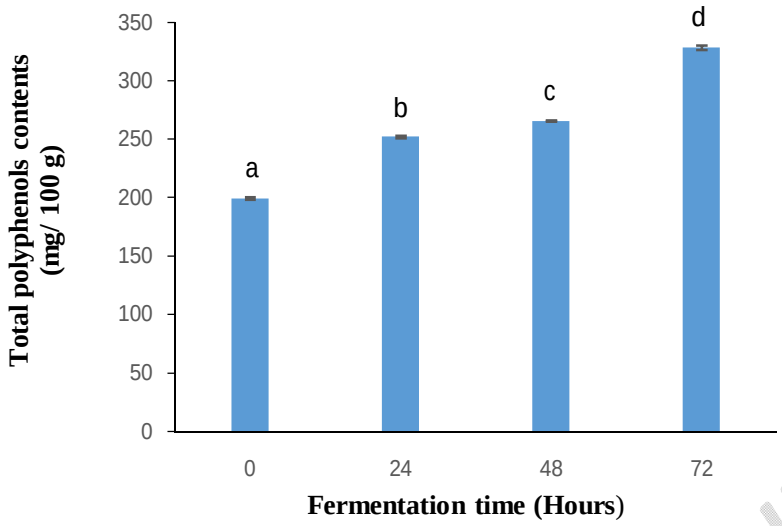

Fig. 2.Changes in the total polyphenol content of bean flour (*Phaseoluslunatus*) during controlled fermentation

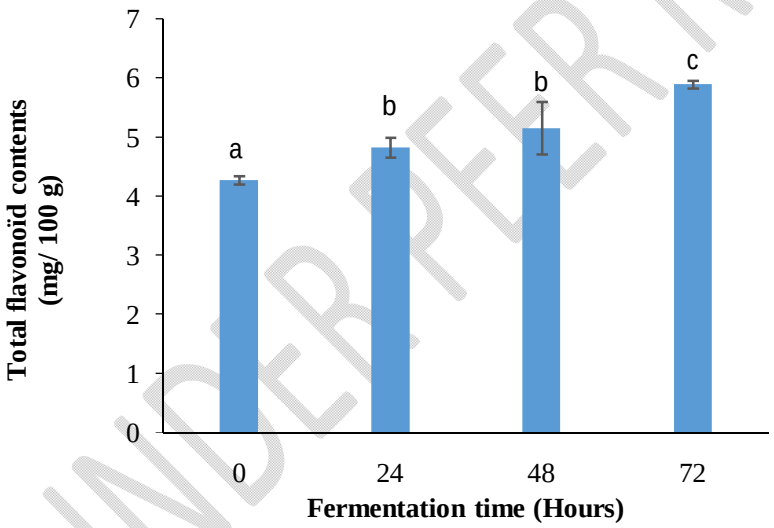

Fig. 3.Changes in the total flavonoid content of bean flour (*Phaseoluslunatus*) during controlled fermentation

3.5. 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 ± 0.17%. The DPPH radical inhibition percentages of phenolic extracts from fermenting flour are shown in Fig. 4. They increased significantly ($p < 0.05$) during fermentation from 75.68 ± 1 .06% to 89.63 ± 0.96% between 0 and 72 hours.

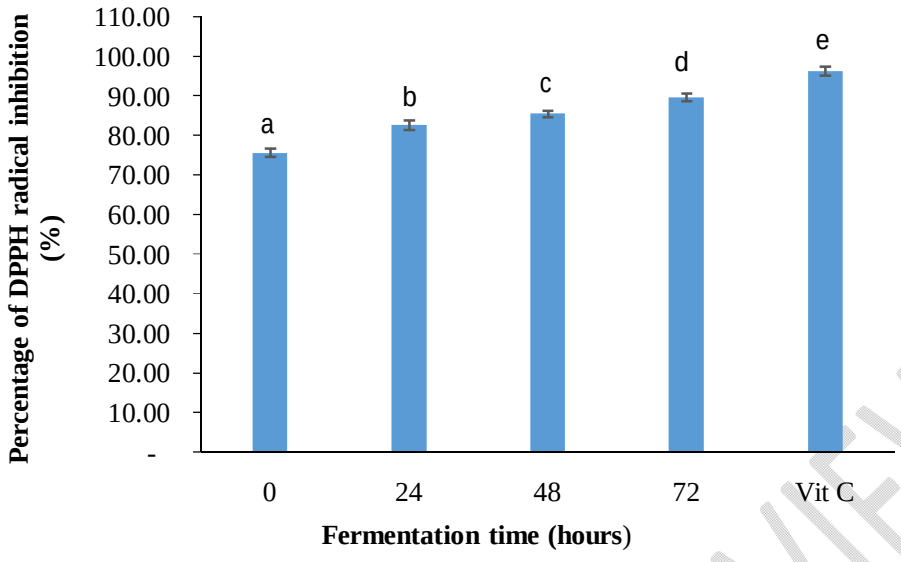

Fig. 4.Percentage inhibition of the DPPH radical in phenolic extracts of bean (*Phaseoluslunatus*) seed flour during controlled fermentation

3.6. Correlation between total polyphenol and total flavonoid contents and antioxidant activity.

The correlation between the contents of total polyphenols and flavonoids and the percentages of inhibition of the DPPH radical and hydrogen peroxide in the methanolic extracts of flour during controlled fermentation was expressed by Pearson's correlation coefficient (r) (Table 4). The contents of total polyphenols and flavonoids were strongly correlated with the percentages of DPPH radical inhibition. Fig. 5 and 6 illustrate the correlations between total polyphenol and flavonoid content and DPPH radical inhibition percentages.

Table 4. Correlation between the contents of total polyphenols, total flavonoids and antioxidant activity

	DPPH
Total polyphenol content	0.962
Total flavonoid content	0.912

Value of r extracted from Pearson correlations between the content of bioactive compounds and the percentage of DPPH radical inhibition.

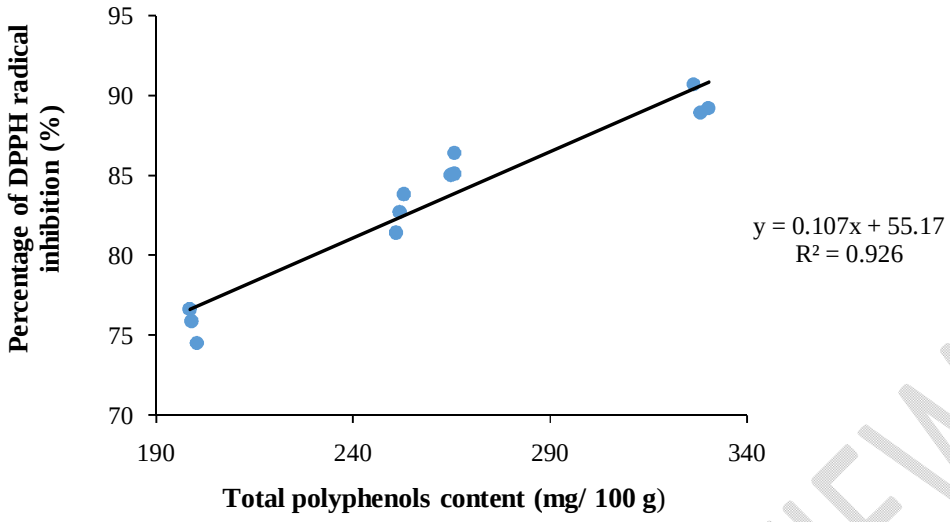

Fig. 5.Correlation between antioxidant activity and total polyphenol contents

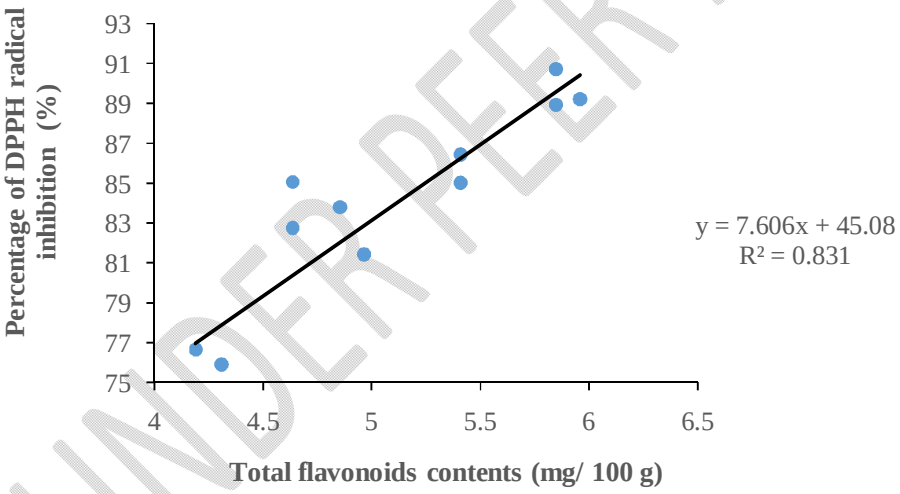

Fig. 6.Correlation between antioxidant activity and total flavonoid contents

4. DISCUSSIONS

Fermentation is a desirable process of biochemical modification of the primary food matrix caused by micro-organisms and their enzymes [7]. 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 [25].

During controlled fermentation of *Phaseoluslunatus* (L) bean flour, protein content increased significantly ($p < 0.05$). This result is consistent with that of Balogun et al. [26] who observed an increase in the protein content of *Prosopisafricana* seeds fermented with *Bacillus subtilis* during their work on the comparative study of the physico-chemical analysis of *Prosopisafricana* seeds fermented with different starter cultures. 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 [27] into the bean substrate by organisms during fermentation with the aim of using the bean starch as a carbon source [28].

Furthermore, the increased growth and proliferation of the microbial biomass could also be responsible for the increase in protein content in the bean substrate [29]. In addition, the lipid content of *Phaseoluslunatus* L. flour dropped significantly ($p < 0.05$) after inoculation with the *Bacillus subtilis* starter. This result is consistent with that of Hu et al. [30] 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. [31] also observed an increase in the lipid content of cooked *Phaseoluslunatus* L. seeds fermented with *Bacillus subtilis* and *Bacillus pumilus*.

Ash levels decreased significantly ($p < 0.05$) in *Phaseoluslunatus* (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 [32]. This result is consistent with that of [30] who observed a reduction in the ash content of black soya during fermentation by *Bacillusnatto*. However, it differs from that of Adegbehingbe et al. [31] who observed an increase in the ash content of cooked *Phaseoluslunatus* (L.) seeds fermented with *Bacillus subtilis*. Jeff-Agboola and Oguntuase [33] 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. [31] who studied the effect of controlled fermentation by *Bacillus subtilis* and *Bacillus pumilus* on the nutrient and antinutrient content of cooked bean seeds (*Phaseoluslunatus* L.). In their study, it was observed that the carbohydrate content of *Phaseoluslunatus* (L.) seeds fermented with *Bacillus subtilis* decreased until the fifth day of fermentation.

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. [34] 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*.

In addition, the total sugar content decreased significantly ($p < 0.05$) during fermentation. This reduction is similar to that observed in Prosopisafricana seeds fermented with a *Bacillus subtilis* starter according to the work of Balogun et al. [26] on the comparative study of the physicochemical analysis of *Prosopisafricana* 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. [35].

They observed an increase in soluble reducing sugars during the first two days of fermentation (2.0-11.0 mg g-1), followed by a decrease during the controlled fermentation of *Prosopisafricana* 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.

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 [36]. 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. [37] 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 *Phaseoluslunatus* (L.) flour by increasing protein bioavailability.

In the body, the phytate molecule is capable of forming insoluble complexes with essential divalent cations such as Fe2+, Zn2+, Mg2+ and Ca2+, etc., thus preventing their bioavailability. In addition, the phytate molecule has the ability to form complexes with proteins and starch, inhibiting their digestion [38]. Their high presence in a food significantly affects its nutritional quality. The phytic acid content of *Phaseoluslunatus* (L.) flour fell significantly during the fermentation period. Yasar and Tosun [39] 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 [40].

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 [41]. 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 [42, 43]. 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. [31], 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 α-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 [44]. 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 [45]. 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. [31], who observed a reduction in hydrocyanic acid content in cooked bean seeds fermented with *Bacillus subtilis* compared with unfermented cooked seeds.

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. [46] who observed an increase in sodium content during fermentation of *Parkiabiglobosa* 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 [47, 48]. 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 *Phaseoluslunatus* a good source of magnesium, with a daily intake of 400 mg/day [49]. 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. [46] also observed an increase in phosphorus content during fermentation of *Parkiabiglobosa* seeds. Fermentation is therefore beneficial for the phosphorus intake of *Phaseoluslunatus*, whose requirements are estimated at 700 mg/day for adults [50]. 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 [51]. During fermentation of *Phaseoluslunatus* (L.) flour by *Bacillus subtilis*, an increase in potassium content was observed. This result is similar to that of Atere et al. [46], who also observed an increase in the potassium content of *Parkiabiglobosa* seeds during fermentation. *Bacillus subtilis*-fermented flour from *Phaseoluslunatus* (L.) is a good source of potassium, with a daily intake of 4.7 mg for adults [47].

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 [52]. 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 *Phaseoluslunatus* (L.). Iron is an essential trace element that plays a vital

role in oxygen transport and energy metabolism [53]. 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 *Phaseoluslunatus* (L.). In fact, the acceptable daily intake is 8 to 18 mg/day for adults [54] 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 [55]. During fermentation, the zinc content of the beans remained constant. The daily zinc intake is 10 mg. *Phasoeluslunatus* (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 [56]. Like zinc, copper levels did not change during fermentation. Fermented *Phaseoluslunatus* (L.) flour could be a good source of copper, with a daily intake for an adult estimated at 1 mg [57].

Antioxidant activity is correlated with the presence of phytochemicals, particularly phenolics, flavonoids and anthocyanins in foods [58]. The content of total polyphenols and total flavonoids increased during fermentation by *Bacillus subtilis*. These results are consistent with those of Yoon et al. [59] who observed an increase in the polyphenol content of rice bran fermented with *Bacillus sutilis* compared with unfermented bran. Juan and Chou [60] also showed that the total polyphenol content of black soybean extract fermented with *Bacillus subtilis* BCRC 14715 was significantly higher ($p< 0.05$) than the respective unfermented black soybean extract. The increase in polyphenol content in the fermented flours could be due to the bioconversion of conjugated forms of phenolic acids into simple forms by the action of the β-glucosidase produced by *Bacillus subtilis*. Indeed, the enzyme β-glucosidase (β-D-glucosideglucohydrolase) catalyses the hydrolysis of glycosidic bonds in alkyl and aryl β-D-glucosides, as well as in glycosides containing only carbohydrate residues [61]. This enzyme has been described as being capable of hydrolysing glycosides to release free phenolic acids [62].

An increase in the potential for inhibition of the DPPH radical and hydrogen peroxide was observed when *P. lunatus* was fermented with *Bacillus subtilis*. Ali et al.[63] reported that the DPPH radical inhibition potential of four soybean cultivars fermented with *Bacillus subtilis* for the production of cheonggkjang was greater than that of unfermented soybeans. Dajanta et al. [64] also reported that naturally fermented soybeans fermented with *Bacillus subtilis* for the production of ' ' thuanao' ' had higher DPPH radical inhibition potency than unfermented soybeans. The high DPPH radical and hydrogen peroxide scavenging potentials are strongly correlated with changes in total polyphenol and total flavonoid contents during controlled fermentation. This strong correlation reflects the involvement of these phenolic compounds in the antioxidant power of fermented flour.

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 (*Phaseoluslunatus*) has improved their nutritional quality. Anti-nutritional factors such as phytates, tannins, oxalate and hydrocyanic acid have been considerably reduced, 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 increased protein content makes fermented beans a good source of protein, which could make *Phaseoluslunatus* (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 subtillis* increases the content of bioactive phenolic compounds and the antioxidant potential by increasing the scavenging potential of the DPPH radical and hydrogen peroxide. Fermentation with *Bacillus subtillis* therefore increases the biological quality of the beans.

REFERENCES

1. Yabile RK**.** Vulnérabilité des Populations à l'Insécurité Alimentaire en Côte d'Ivoire : Cas des Régions du Worodougou, de la Vallée du Bandama, du N'Zi Comoé et du Zanzan, *Agron*. *Afr*. 2011; 23 (2) : 179-192
2. FAO. Traditional food plants. *Food and Nutrition Paper*, 1988 ; 42: 1-3
3. Young VR, Pellett PL. Plant proteins in relation to human protein and amino acid nutrition. Am. J. Clin. Nutr. 1994; 59: 1203S–1212S.
4. Fadahunsi IF. The effect of soaking, boiling and fermentation with *Rhizopusoligosporus* on the water soluble vitamin content of Bambara groundnut. 2009; Pak. J. Nutr.8 (6): 835-840.
5. Ezeagu EI, Ibegbu MD. Biochemical composition and nutritional potential of ukpa: a variety of tropical Lima beans (*Phaseolus lunatus*) from Nigeria. Polish J. Food Nutr. Sci.2010; 60(3), pp. 231-235.
6. Emiola IA, Ologhobo AD, Gous RM. Performance and histological responses of internal organs of broiler chickens fed raw, dehulled, and aqueous and dry-heated kidney bean meals. Poult. Sci. 2007; 86: 1234-1240.
7. Kahajdova Z, Karovicova J. Fermentation of cereals for specific purpose. J. Food Nutr. Res. 2007; 46: 51- 57.
8. Hotz C, Gibson RS. Traditional food-processing and preparation practices to enhance the bioavailability of micronutrients in plants-based diets. J. Nutr. 2007; 137: 1097 - 1100.
9. Steinkraus KH. Nutritional significance of fermented foods. Food Res. Int. 1994; 21: 259–267.
10. Chaves-Lopez C, Serio A, Grande-Tovar CD, Cuervo-Mulet R, Delgado-Ospina J, Paparella A. Traditional fermented foods and beverages from a microbiological and nutritional perspective: The Colombian Heritage. Compr. Rev. Food Sci. Food Saf. 2014; 13: 1031 – 1048.
11. Hasan MN, Sultan MZ, Mar-E-Um M. Significance of Fermented Food in Nutrition and Food Science.J. Sci. Res. 2014;**6**(2):373 - 386.
12. Laranjo M. Starter cultures and their role in fermented foods. Microbial fermentations in Nature and as Designed Processes, 2023;p. 281-292
13. Konkol D, Jonuzi E, Popiela E, Sierzant K, Korzeniowska M, Leich K, Gumowski M, Krasowska A, Lukaszewicz M, Korczynski M. Influence of solid state fermentation with *Bacillus subtilis* 67 strain on the nutritional value of rapeseed meal and its effects on performance and meat quality of broiler chickens. Poult. Sci. 2023; 107 (7): 1-7.
14. Sanjukta S, Rai AK.Production of bioactive peptides during soybean fermentation and their potential health benefits. Trends Food Sci. Technol. 2016; 50: 1 - 10.
15. AOAC. Official methods of analysis. Method 925.45. Inc. Arlington, VA, USA: Association of Official Analytical Chemists. 1990

16. Muller HG, Tobin G. Nutrition and Food Processing. The Avi Pub Co, Wesport Connecticut,USA, 1980; p. 302.
17. Dubois M, Gilles KA, Hamilton JD, Rebers RA, Smith M. Colometric method for determination of sugar and related substance. Anal. Chem. 1956; 28: 350 - 356.
18. Bernfeld P. Amylase α and β methods in enzymology 1. S. P. colowick and N.O. K, edition academic press, Inc New-York, 1955; p 149-154.
19. Bainbridge Z, Tromlins K, Westby A.Analysis of condensed tannins using acidified vanillin. J. Sci. Food Agric. 1996; 29: 77 – 79
20. Latta M, Eskin M.A simple method for phytate determination. J. Agric. Food Chem. 1980; **67**: 1313 – 1315.
21. Day RA, Underwood AL. Quantitative Analysis. 5th Edition, Prentice Hall Publication, Upper Saddle River, 1986; 701p.
22. Singleton VL, Orthofer R, Lamuela-Raventos RM. Analysis of total phenols and other oxidant substrates and antioxidants by means of Folin-Ciocalteu reagent.Meth. Enzymol.1999; 299: 152-178.
23. Meda AL, Lamien CE, Romito M, Millogo J, Nacoulma OG. Determination of the total phenolic, flavonoid and proline Contents in Burkina Fasan Honey, as well as their radical scavenging activity. Food Chem. 2005; 91(3):571-577
24. Hatano T, Kagawa H, Yasuhara T, Okuda T. Two New Flavonoids and Other Constituents in Licorice Root: Their Relative Astringency and Radical Scavenging Effects. Chem. Pharm. Bull. 1988; 36: 2090–2097.
25. Kimura K. & Yokoyama S. (2019). Trends in the application of *Bacillus* in fermented foods. *Current Opinion in Biotechnology*, 56: 36 – 42.
26. Balogun MA, Oyeyiola GP, Kolawole FL. Comparative study of physicochemical analysis of *prosopisafricana*seeds fermented with different starter cultures. Croat. j. food sci. technol.2017; 9 (1): 25 - 30
27. Oboh G, Akindahunsi AA. Biochemical changes in cassava products (flour and gari) subjected to *Saccharomyces cerevisae* solid media fermentation. Food Chem. 2003;82: 599–602.
28. Raimbault M. General and microbiological aspects of solid substrate fermentation, Electron. J. Biotechnol. 1998; 3: 1 – 20.
29. Oboh G. Nutrient enrichment of cassava peels using a mixed culture of *Saccharomyces cerevisiae* and *Lactobacillus* spp. solid media fermentation techniques. Electron. J. Biotechnol, 2006; 9 (1): 46 - 49.
30. Hu Y,Ge C, Yuan W, Zhu R, Zhang W, Du L, Xue J.Characterization of fermented black soybean natto inoculated with *Bacillus natto* during fermentation. J. Sci. Food Agric. 2010; 90 (7): 1194 – 1202.
31. AdegbehingbeKT, AdetuyiFC, AkinyosoyeFA.Effect of Fermentation on Nutrient and Anti-Nutrient Contents of Ground-Cooked Lima Bean (*Phaseolus lunatus*) Seeds using *Bacillus subtilis*and *Bacillus pumilus*. Br. Microbiol. Res. J.2014; 4(11): 1285-1298.
32. Reebe S, Gonzalez VN, Rengifo J. Research on trace elements in the common beans. Food Nutr Bull. 2000; 21: 387 - 391.
33. Jeff-Agboola YA, Oguntuase OS. Effect of *Bacillus sphaericus* on proximate composition of soybean (*Glycine max*) for the production of soy Iru. Pak. J. Nutr.2006; **5** (6): 606- 607.
34. Chu J, Zhao H, Lu Z, Lu F, Bie X, Zhang C. Improved physicochemical and functional properties of dietary fiber from millet bran fermented by *Bacillu*s natto.Food Chem.2019; 294: 79–86.
35. Ogunshe AAO, Omotosho MO, Ayansina ADV.Microbial Studies and Biochemical Characteristics of Controlled Fermented *Afiyo*- a Nigerian Fermented Food Condiment from *Prosopisafricana*(Guill and Perr.) Taub. Pak J. Nutr. 2007; 6 (6): 620 - 627

36. Smeriglio A, Barreca D, Bellocco E, Trombetta D. Proanthocyanidins and hydrolysable tannins: occurrence, dietary intake and pharmacological effects. Br. J. Pharmacol.2016; 174 (11): 1244- 1262.
37. Sharma A, Kumari S, Wongputtisin P, Nout MJR, Sarkar PK. Optimization of soybean processing into kinema, a *Bacillus*-fermented alkaline food, with respect to a minimum level of antinutrients.J. Appl. Microbiol.2015;119 (1): 162–176.
38. Nissar J, Ahad T, Naik HR, Hussain SZ. A review phytic acid: As antinutrient or nutraceutical.J. pharmacogn. phytochem. 2017;6 (6): 1554 - 1560
39. Yasar S, Tosun R. Increased organic acid production and decreased phytate phosphorus by high level of water content of *Bacillus subtilis* ATCC PTA-6737 fermentation of soybean meal. Bulletin of University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca. Animal Science and Biotechnologies, 2019; 76 (1): 1 – 6.
40. Hirvonen J, Liljavirta J, Saarinen MT, Lehtinen MJ, Ahonen I, Nurminen P.Effect of Phytase on In Vitro Hydrolysis of Phytate and the Formation of myo-Inositol Phosphate Esters in Various Feed Materials.J. Agric. Food Chem. 2019; 67(41): 11396-11402.
41. Mitchell T, Kumar P, Reddy T, Wood KD, Knight J, Assimos DG, Holmes R. P.Dietary oxalate and kidney stone formation. Am. J. Physiol. Renal Physiol.2019; 316: F409–F413.
42. Schenck PA, Chew DJ, Nagode LA, Rosol TJ. Disorders of Calcium: Hypercalcemia and Hypocalcemia. Fluid, Electrolyte, and Acid-Base. Disorders in Small Animal Practice, 2006; pp. 122–194.
43. Rahman MM, Abdullah RB, Wan Khadijah WE.A review of oxalate poisoning in domestic animals: tolerance and performance aspects. J. Anim. Physiol. An. N. 2012; 97(4): 605–614.
44. Vetter J.Plant cyanogenic glycosides. *Toxicon,* 2000; 38 (1): 11–36.
45. Codex alimentarus Commission. Repport of the eigth session of the codex coordinating committee for Africa. FAO/WAO, Cairo Egypt. 1988; 123p
46. Atere AV, Oyetayo VO, Akinyosoye FA. Effect of Different Fermentation Time on the Microbial, Mineral and Proximate Composition of *Parkiabiglobosa* Seeds Fermented for Iru Production. Sustain. Food Prod.2020; **7**: 17-23
47. Karppanen H, Karppanen P, Mervaala E. Why and how to implement sodium, potassium, calcium, and magnesium changes in food items and diets? J. Hum. Hypertens. 2005; 19 (3): S10 - 9.
48. Strazzullo P, Leclercq C.Sodium. AdvNutr, 2014; 5 (2): 188- 190
49. Costello R, Wallace TC, Rosanoff A. Magnesium. AdvNutr, 2016; **7** (1): 199 - 201.
50. Calvo MS, Lamberg-Allardt CJ. Phosphorus. AdvNutr, 2015; 6 (6): 860 - 862.
51. Yin J, Hu Y, Yoon J.Fluorescent probes and bioimaging: alkali metals, alkaline earth metals and pH.Chem. Soc. Rev.2015; 44(14): 4619 – 4644.
52. Kiela PR, Ghishan FK. Physiology of intestinal absorption and secretion. Best Pract. Res. Clin. Gastroenterol. 2016; 30(2): 145– 159.
53. Cook JD. Parenteral trace elements: iron. Bulletin of the New York Academy of Medecine. 1984; 60 (2):156 - 162.
54. Finnamore HE, Whelan K, Hickson M, Shovlin CL. Top dietary iron sources in the UK. British Journal of Genetic Practice, 2014; 64 (621): 172- 173.
55. Huang L, Drake VJ, Ho E. Zinc. AdvNutr, 2015; 6(2): 224 - 6.
56. Ma J, Betts NM. Zinc and copper intakes and their major food sources for older adults in the 1994-96 continuing survey of food intakes by individuals (CSFII). J. Nutr. 2000; 130 (11): 2838 - 2843.

57. Livingstone C. Review of Copper Provision in the Parenteral Nutrition of Adults [Formula: see text]. Nutr Clin Pract, 2017; 32(2): 153-165.
58. Kadiri O.A Review on the Status of the Phenolic Compounds and Antioxidant Capacity of the Flour: Effects of Cereal Processing. Int. J. Food Prop.2017;**20** (1):798 - 809
59. Yoon HJ, Lee KA, Lee JH, Jin HJ, Kim HJ, Kim K.-T, Paik H-D.Effect of fermentation by *Bacillus subtilis* on antioxidant and cytotoxic activities of black rice bran. Int. J. Food Sci. Technol.2014; 50 (3): 612 - 618.
60. Juan M-Y, Chou C-C.Enhancement of antioxidant activity, total phenolic and flavonoid content of black soybeans by solid state fermentation with *Bacillus subtilis* BCRC 14715.Food Microbiol.2010; 27 (5): 586–591.
61. Vattem DA, Shetty K. Ellagic acid production and phenolic antioxidant activity in cranberry pomace (*Vaccinium macrocarpon*) mediated by *Lentinus edodes* using a solid-state system. Process Biochem, 2003; 39: 367-379
62. Zheng Z, Shetty K. Solid state production of polygalacturonase by 316 *Lentinus edodes* using fruit processing wastes. Process Biochem, 2000; 35: 825- 317 830.
63. Ali MW, Shahzad R, Bilal S, Adhikari B, Kim Il-D, Lee J-D, Lee I-J, Kim BO, Shin D-H.Comparison of antioxidants potential, metabolites, and nutritional profiles of Korean fermented soybean (Cheonggukjang) with *Bacillus subtilis* KCTC 13241. Food Sci. Technol.2018; 55 (8): 2871–2880.
64. Dajanta K, Apichartsrangkoon A, Chukeatirote E. Antioxidant Properties and Total Phenolics of Thua Nao (a Thai Fermented Soybean) as Affected by *Bacillus*-fermentation. J. Microb. Biochem. Technol. 2011;3(4): 056 – 059.