Original Research Article

Effect of fermentation using *Bacillus subtilis* ATCC 6051 on nutrients, antinutrients and antioxidant potential of red black bean seed flour (*Phaseolus Lunatus*)

ABSTRACT

Objective: Lima bean (*Phaseolus lunatus*) is a species of legume rich in protein, but little consumed because its consumption causes a digestion problem due to the high content of anti-nutritional factors. Thus, the objective of this study is to promote this legume by improving its nutritional and antioxidant quality through controlled fermentation with *Bacillus subtilis* ATCC 6051.

Methodology and results: The impact of fermentation time with *Bacillus subtilis* on the proximal composition, antinutritive factors, bioactive phenolic compounds and antioxidant capacity of lima bean flour were evaluated. The protein and fiber contents increased during fermentation (19.77 to 22.93 %) and (5.99 to 8.28 %) while the lipid and carbohydrate contents decreased. The levels of antinutritional factors decreased significantly ($p< 0.05$). The contents of bioactive phenolic compounds as well as the antioxidant power increased significantly ($p< 0.05$) during fermentation. A strong correlation between the evolution of the contents of bioactive compounds and the antioxidant potential was observed.

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 an ingredient in human food.

Keywords: Lima bean, fermentation, nutrient, anti-nutrition factors, antioxidant activity, *Bacillus sutilis*

1. INTRODUCTION

The majority of the African population faces the problem of undernourishment [1]. The consumption of legumes seems to be an alternative to this problem [2]. Of these legumes, *Phaseolus lunatus* (L) is a species of bean that belongs to the Fabaceae family. This species of bean is very rich in phytochemicals and proteins. The protein richness of the Lima bean (*Phaseolus lunatus*) could also compensate for the increasing protein deficiency in Africa given the high price of animal proteins [3]. However, the consumption of these legumes poses a problem with digestion and bioavailability of certain nutrients due to the high content of anti-nutritional factors [4]. Lima bean seeds contain antinutritional factors such as hydrogen cyanide, phytic acids, saponin, oxalate, tannin, trypsin inhibitory activity, agglutinate activity [5]. These anti-nutritional factors have been observed to inhibit the absorption of nutrients and their subsequent utilization and assimilation by animals. In addition, they cause damage to certain organs such as the liver, kidneys and spleen [6]. It is therefore important to find ways to overcome this problem.

Fermentation is a desirable process of biochemical modification of the primary food matrix caused by microorganisms and their enzymes [7]. Fermentation is used to improve the bioaccessibility and bioavailability of nutrients [8]and improves organoleptic properties as

well as extension of shelf life [9, 10, 11]. The fermentation of a food can be done 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 microorganisms, but also by producing bacteriocins that inhibit the growth of unwanted microbes. Additionally, through their control of food microbiota, starter cultures can extend the shelf life of fermented foods. In addition, they promote certain organoleptic characteristics of fermented foods, namely their color, their aroma, their flavor or even their texture [12]. Some indigenous legume-based fermented foods in Asia and Africa have benefited from technological advances through the use of *Bacillus subtilis* strains as starter cultures. Studies have shown the beneficial impact that the use of *Bacillus subtilis* for fermentation has had on the nutritional quality of rapeseed [13]. Furthermore, studies have shown that certain traditional foods of Asian origin produced from legumes fermented with *Bacillus subtilis* have various health benefits such as antihypertensive, antidiabetic and anticancer properties [14]. Also, *Bacillus subtilis* strains are used to produce a range of fermented foods, including sauerkraut, pickles, olives, vinegar, dairy and other products. Thus, the objective of this study is to show the effect of controlled fermentation with *Bacillus subtilis* on the nutritional quality and antioxidant power of lima bean.

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 red-spotted cultivar of *Phaseolus lunatus* (L.) (Fig. 1). A producer in the villages of Assoumoukro (M'batto) and N'guessankro (Bongouanou), the central-eastern park of Côte d'Ivoire, supplied it. The dried bean seed samples were cleaned and sorted according to size and absence of extraneous or abnormal odours and living or dead insects. The bean samples were finally ground in a suitable analytical mill and sieved through a 0.5 mm mesh sieve.

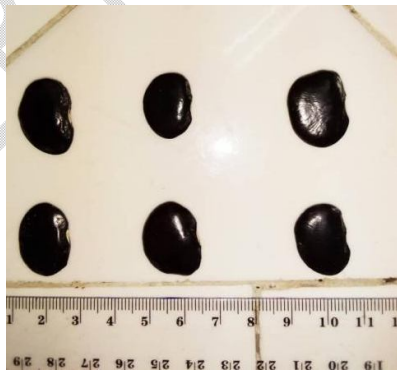

Fig. 1.Mature seeds of the black red-spotted cultivar of *Phaseolus lunatus* (L.)

2.2. Fermentation experiments

Approximately 200 g of bean flour were transferred into 4 flat-bottomed flasks of 500 mL each and autoclaved at 121 °C for 15 min. The sterilized 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 bean 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 sifted through a 0.25 mm mesh sieve. Then the flours obtained were stored in glass jars at room temperature until used [4].

2.3. Proximate Analysis

The different samples produced at different fermentation times (0, 24, 48, 72 hr) were analyzed for ash, crude fat, crude protein and crude fiberin proportions of 1 g each, by standard methods recommended by [15]. Carbohydrates were calculated by the difference based on the total composition of fermented and unfermented flours [16].

The total sugars were determined according to the technique described by [17] using phenol and concentrated sulfuric acid. The ethanosoluble extract (150 µL) was taken and placed in a test tube. Then, 1 mL of phenol (5%, w / v) and 1 mL of concentrated sulfuric acid (97%) were added. The reaction medium was homogenized and left 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 ethanosoluble extract. The optical density was converted into the amount of total sugars using a standard curve obtained from a glucose solution (1g / L).

The reducing sugars were determined according to the method of [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 were added. The mixture was brought to a boiling water bath for 5 min. 2 mL of distilled water were 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 amount 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 using atomic absorption spectrophotometry as described by the method of [15].

2.5. Anti-nutritional factors estimation

Hydrogen cyanide was analysed by the [15]method. Ten (10) g of flour were homogenized 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 from 20 mL of sodium hydroxide (0.1 N) and 2 mL of KI (0.02 N). The distillate was titrated with silver nitrate $AgNO_3$ (0.02 N) until a yellowish haze appears.

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 by[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 [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 of lima bean flour (fermented and unfermented) were measured according to the method described by [22]. One milliliter (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 whole 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, replacing the folin-ciocalteu reagent with 70% (v/v) methanol. A standard range was established from a stock solution of gallic acid (1 mg/mL) under the same conditions as the test and made it possible to determine the quantity of phenols in the sample. The dosage of flavonoids was determined according to the method described by [23]. A volume of 0.5 mL of methanolic extract was introduced into a test tube. To the contents were added successively 0.5 mL of distilled water, 0.5 mL of aluminum chloride (10%, w/v), 0.5 mL of sodium acetate (1 M) and 2 mL of distilled water. The blank was prepared for each sample, by adding 0.5 mL of distilled water to the test tubes replacing the methanolic extract. The tubes were left to stand for 20 min in the dark and the absorbance was read with a spectrophotometer at 415 nm against a blank. A standard range was established from a solution of quercetin at (0.1 mg/mL) under the same conditions as the test.

2.7. Evaluation of antioxidant activity

The potential for trapping the free radical DPPH was evaluated on fermented and non-fermented flours having undergone hydromethanolic maceration. The capacity to trap “stable” DPPH free radicals of crude extract from each of the different flour samples was monitored according to the method of [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 left to stand for 30 min in the dark. The reduction in DPPH was determined by measuring the 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 of each of the samples, translated by the decolorization of the DPPH solution, was calculated as a percentage of inhibition using the following equation:

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

$A_{control}$ = Absorbance of methanolic DPPH solution (3 mM)
A_{sample} = Absorbance of DPPH solution reduced by sample extract

2.8. Statistical analysis

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

3. RESULTS

3.1.Biochemical composition

The biochemical composition of Lima bean (*Phaseolus lunatus*) seed flour at different fermentation times controlled with *Bacillus subtilis* is presented in Table 1. The protein content increased significantly ($p< 0.05$) during fermentation going from 19.77 ± 0.10% to 22.93 ± 0.16% after 72 hours. Unlike the protein content, the lipid content decreased during fermentation. From 0 to 72 hours of fermentation, it varied from 1.67 ± 0.06 to 1.19 ± 0.05. The fiber content increased during the controlled fermentation of 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. The carbohydrate content decreased significantly ($p< 0.05$) during fermentation. It went from 68.57 ± 0.14% to 64.41 ± 0.09%. The total sugar content decreased during controlled fermentation. Indeed, the variation was significant ($p< 0.05$) from 0 to 72 hours with respective values of 4.18 ± 0.02 and 2.16 ± 0.01 g/100 g. The reducing sugar content experienced a bell-shaped evolution with an increase from 0 to 48 hours and a decrease beyond 48 hours. Thus, from 0 to 48 hours, the increase was significant ($p< 0.05$) with respective values of 39.45 ± 0.42 mg/100 g and 83.58 ± 0.45 mg. / 100 g and from 48 to 72 hours, the decrease was also significant ($p< 0.05$) with respective values of 83.58 ± 0.45 and 43.51 ± 0.58 mg/ 100.

Table 1.Evolution of biochemical composition of Lima bean seed flour during the fermentation with *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± 0.07c[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)

3.2. Mineral composition

The mineral composition of Lima bean seed flour at different controlled fermentation times is shown inTable 2. Before inoculation of the *Bacillus subtilis* starter into the flour, the sodium concentration (Na) was 15.8 ± 0.2 mg/100 g. After inoculation of the *Bacillus subtilis* starter, it gradually increased to reach the 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. As for the magnesium (Mg) content, it 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. Furthermore, the P content decreased significantly in the first 24 hours following inoculation of *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 *Phaseolus lunatus* flour increased significantly ($p< 0.05$) as a function of 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. Like K, Ca content increased significantly ($p< 0.05$) after inoculation of *Bacillus subtilis* into bean flour. It varied to reach the value of 148.9 ± 1 mg/100 g at 72 hours. Concerning the Fe content, it decreased from 12.9 ± 0.1 mg/ 100 g to 7.3 ± 0.4 mg/ 100 g. Unlike other minerals which varied during fermentation, the Cu and Zn contents did not vary. They remained at 0.5 ± 0.1 mg/ 100 g and 2.4 ± 0.1 mg/ 100 g, respectively.

Table 2.Evolution of mineral contents (mg/ 100g)of Lima bean seed flour during the 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 ($p<0.05$)

3.3. Antinutritional factors

The composition of antinutritional factors of Lima bean seed flour at different fermentation times is shown in Table 3. The analysis of the results showed that the contents of antinutritional factors decreased significantly ($p< 0.05$) during the controlled fermentation. Thus, the tannin content drops during 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. The oxalate content also decreased significantly ($p< 0.05$) during fermentation. It varied from 221.83 ± 3.17 mg/100 g to 171.41 ± 1.59 mg/100 g. Like all other antinutritional factors, the hydrocyanic acid content decreased throughout the fermentation time. This reduction is 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.Evolution of the content (mg/ 100g) of antinutritional factors in bean (*Phaseolus lunatus*) seed flour during fermentation with *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 ($p< 0.05$).

3.4.Bioactive phenolic compounds

The changes in the polyphenol and flavonoid contents during fermentation are presented respectively in Fig. 2 and 3. The analysis of the results showed significant variations ($p< 0.05$) during their evolution. Thus, 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. The content of total flavonoids also increased like that of total polyphenols. It variedfrom 4.27 ± 0.07 to 5.89 ± 0.06 between 0 h and 72 h.

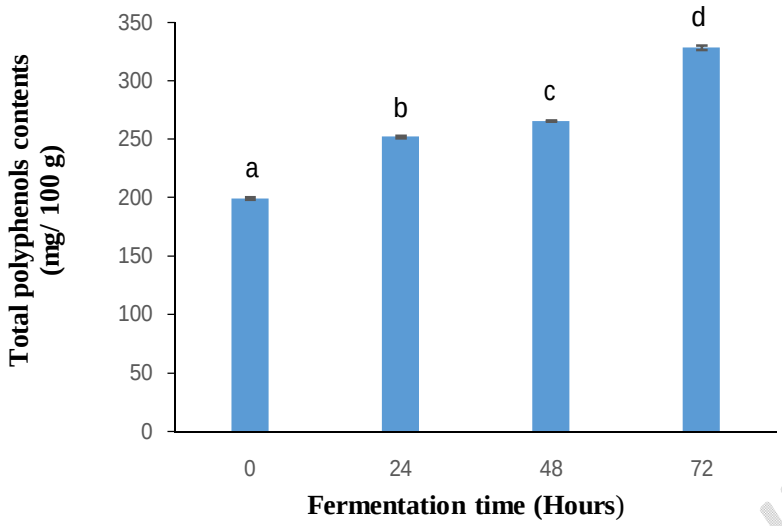

Fig. 2.Evolution of the total polyphenol content of bean (*Phaseolus lunatus*) seed flour during controlled fermentation

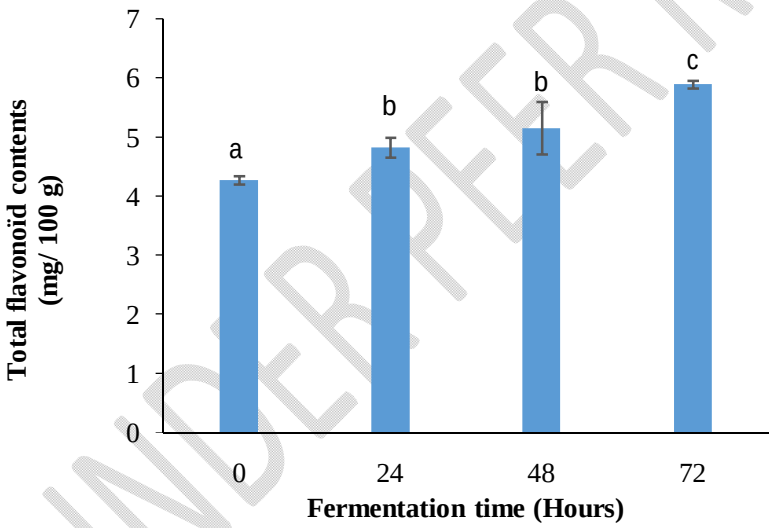

Fig. 3.Evolution of the total flavonoid content of bean (*Phaseolus lunatus*) seed flour during controlled fermentation

3.5. Antioxidant activity

The phenolic extracts of flour during controlled fermentation presented percentages of DPPH radical inhibition greater than 50% at the concentration of 4 mg/ mL in the extraction solvent. They were lower than that of vitamin C which had a value of 96.03 ± 0.17%. The percentages of inhibition of the DPPH radical of flour phenolic extracts during fermentation are highlighted 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.

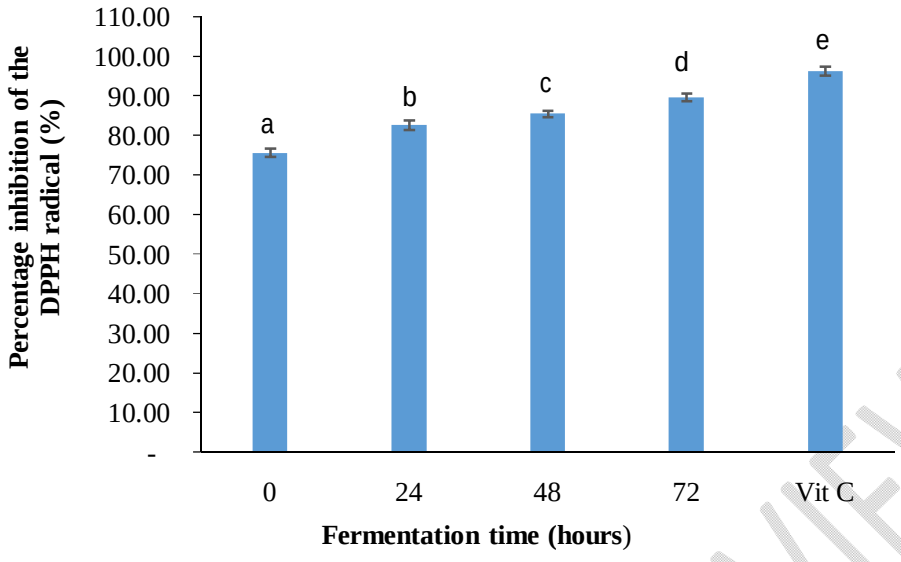

Fig. 4.percentage of inhibition of the DPPH radical of phenolic extracts of bean(*Phaseolus lunatus*) seed flour during controlled fermentation

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

The correlation between the contents of total polyphenols, flavonoids and the percentages of inhibition of the DPPH radical and hydrogen peroxide of the methanolic extracts of the flour during the controlled fermentation was expressed with the Pearson correlation coefficient (r) (Table 4). The contents of total polyphenols and flavonoids are strongly correlated with the percentages of inhibition of the DPPH radical. Fig. 5and6 present an illustration of the correlations between the contents of total polyphenols, flavonoids and the percentages of inhibition of the DPPH radical.

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 contents of bioactive compounds and the percentage of inhibition of the DPPH radical.

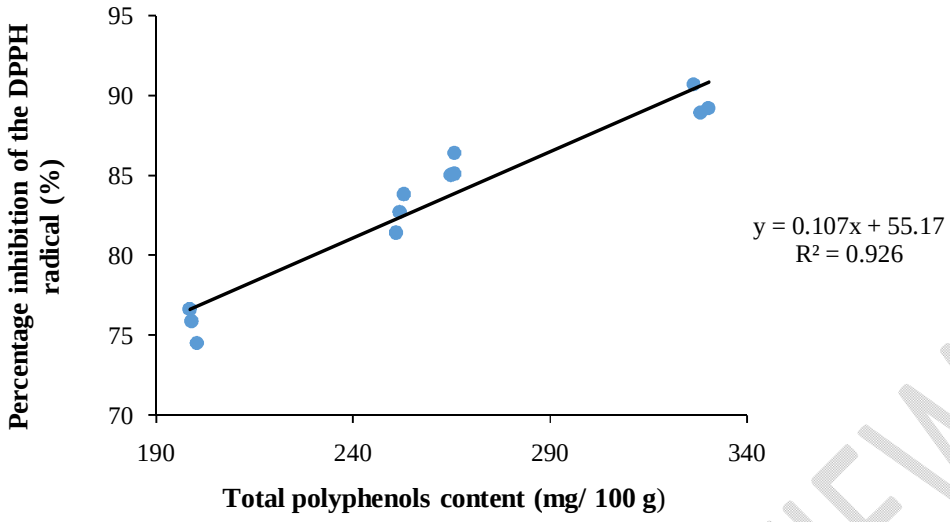

Fig. 5.Correlation between antioxidant activity and total polyphenol contents

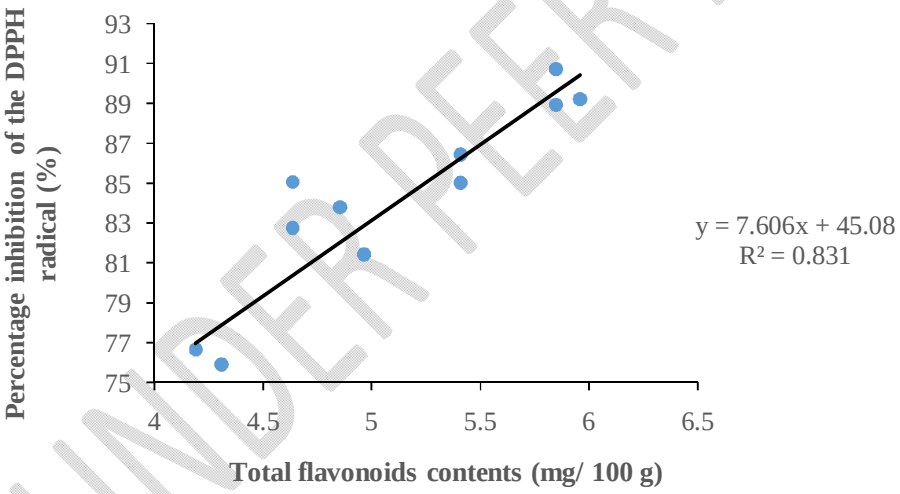

Fig. 6.Correlation between antioxidant activity and total flavonoid contents

4. DISCUSSIONS

Fermentation is a desirable process of biochemical modification of the primary food matrix caused by microorganisms and their enzymes [7]. Controlled fermentations are used to produce a range of fermented foods. *Bacillus* species such as *Bacillus subtilis* and *Bacillusamyloliquefaciens* are widely used to produce fermented foods from soybeans and carobs in Asia and West Africa respectively [25].

During the controlled fermentation time of *Phaseolus lunatus* bean flour (L), the protein content increased significantly ($p< 0.05$). This result is consistent with that of [26] who observed an increase in the protein content of *Prosopis africana* seeds fermented with *Bacillus subtilis* during their work on the comparative study of the physicochemical analysis of *Prosopis africana* seeds fermented with different starter cultures. The increased 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 in an attempt to use bean starch as a carbon source [28]. Furthermore, the increased growth and proliferation of microbial bio mass could also be responsible for the increased protein content in the bean substrate [29]. Furthermore, the lipid content of *Phaseolus lunatus* L. flour dropped significantly ($p< 0.05$) after inoculation of *Bacillus subtilis* starter. This result is consistent with that of [30] who studied the characterization of natto produced from black soybean fermented with *Bacillus subtilis* (natto) during fermentation. The drop in lipid content could result in the hydrolysis of lipids by lipases produced by strains of *Bacillus subtilis* which will hydrolyze triglycerides into fatty acids, sterols during the fermentation process. On the other hand, [31] observed an increase in the lipid content of cooked seeds of *Phaseolus lunatus* L. fermented by *Bacillus subtilis* and *Bacillus pumilus.*

The ash rate decreased significantly ($p< 0.05$) in *Phaseolus lunatus* (L.) seed flours during their fermentation controlled by *Bacillus subtilis*. This reduction could be due to the use of inorganic salts by microbial strains for their metabolism [32]. This result is consistent with that of [30] who observed a decrease in the ash content of black soybeans during fermentation with *Bacillus natto*. However, it is different from that of [31] who observed an increase in the ash content of cooked seeds of *Phaseolus lunatus* (L.) having undergone fermentation by *Bacillus subtilis*. Also, [33] observed an increase in the ash content of soybean seeds fermented by *Bacillus sphaericus* for the production of iru. They attributed the increase in ash content to microbial destruction of anti-nutritional factors which would underlie the increase in certain minerals.

The carbohydrate content of *Bacillus subtilis*-inoculated bean flour decreased during the fermentation process. This decrease in carbohydrate content could be due to the use of carbohydrates as energy sources by microorganisms. Indeed, microorganisms could produce enzymes (amylases and maltase) to hydrolyze complex sugars (starch) into simple sugars (glucose) that can be easily used by microorganisms as energy sources. Furthermore, Bacillus subtilis has significant amylase activity. This significant activity could be the cause of this reduction in carbohydrate content. This result is in agreement with that of [31] who studied the effect of controlled fermentation by *Bacillus subtilis* and *Bacillus pumilus* on the nutrient and anti-nutrient content of cooked bean (*Phaseolus lunatus* L.) seeds. From 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.

Dietary fibers are largely made up of celluloses and hemicelluloses. During flour fermentation, a progressive and significant increase ($p< 0.05$) in its fiber content was observed. This increase in fiber content could be due to the action of enzymes produced by *Bacillus subtilis* to produce easily usable energy sources. Then, these enzymes will degrade

the other macronutrients for the production of these easily usable energy sources, thus increasing the percentage of fiber as the fermentation time increases. [34] also observed an increase in the fiber content of fermented millet bran compared to unfermented bran during their work on improving the physicochemical and functional properties of dietary fiber from millet bran fermented by *Bacillus natto*.

Furthermore, the total sugar content decreases 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 [26] relating to the comparative study of the physicochemical analysis of *Prosopis africana* seeds fermented with different starter cultures. The reduction in total sugars suggested their use as an energy source by *Bacillus subtilis*. Also, there could be a reduction in flatulence oligosaccharides such as stachyose, verbascose and raffinose during the controlled fermentation process. Flatulence oligosaccharides are responsible for intestinal gas after consuming beans. Regarding the reducing sugar content, it increases significantly up to 48 hours following inoculation of *Bacillus subtilis* and then decreases. This evolution of the reducing sugar content is consistent with that reported by [35]. They, in fact, 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 *Prosopis africana* for production. of Afiyo, a traditional African condiment. The increase in reducing sugar content could be due to the action of enzymes produced by microorganisms to produce simple sugars (glucose, fructose). The reduction in content beyond 48 hours would be due to the use of the reducing sugars produced as energy sources.

Tannins are plant substances from the polyphenol family that have the capacity to precipitate macromolecules such as proteins and carbohydrates, leading to the reduction of their bioavailability in a food [36]. They thus reduce the nutritional value of a food because they prevent the digestion of proteins. During the fermentation period, a significant reduction in the tannin content was observed. [37] also observed a decrease in the tannin content of soybean fermented by *Bacillus subtilis* for the production of kinema during their work on optimizing the transformation of soybean into kinema, an alkaline food fermented by *Bacillus*, compared to a minimum level of anti-nutrients. The reduction in tannin content could be explained by the fact that *Bacillus subtilis* has the capacity to secrete tannases which are tannin hydrolytic enzymes. The hydrolysis of tannins leads to the reduction of their content in the substrate. The significant reduction ($p < 0.05$) of tannins during controlled fermentation contributes to the improvement of the nutritional quality of *Phaseolus lunatus* (L.) flour by increasing the bioavailability of proteins.

In the body, the phytate molecule is capable of forming insoluble complexes with essential divalent cations such as Fe^{2+}, Zn^{2+}, Mg^{2+} and Ca^{2+}, etc., thereby preventing their bioavailability. Also, the phytate molecule has the ability to form complexes with proteins and starch, thus inhibiting their digestion [38]. Their strong presence in a food significantly affects its nutritional quality. The phytic acid content of *Phaseolus lunatus* (L.) flour dropped significantly during the fermentation period. [39] observed a reduction in phytate content in soybeans fermented with *Bacillus subtilis* ATCC PTA-6737. This reduction would suggest that *Bacillus subtilis* has the capacity to secrete phytase. The latter is an enzyme that breaks down phytate by dephosphorilating it [40].

Dietary oxalate is of plant origin and can be a component of vegetables, nuts, fruits and grains. In normal individuals, approximately half of urinary oxalate comes from diet and half from endogenous synthesis. Consumption of foods rich in oxalates can cause kidney stones [41]. Furthermore, a diet rich in oxalates can cause hypocalcemia. Serum calcium regulates the synthesis of parathyroid hormone which, in turn, increases tubular calcium reabsorption and increases bone resorption [42, 43]. It therefore appears useful to reduce its content in

foods by treatment. After inoculation of *Bacillus subtilis* into bean flours, the oxalate content of these flours gradually decreased following the duration of inoculation. This result is similar to that of [31] who observed a reduction in oxalate content in cooked bean seeds fermented with *Bacillus subtilis* compared to cooked unfermented seeds. The reduction in oxalate levels could be due to the activity of enzymes produced by microorganisms.

Cyanogenic glycosides can be chemically defined as α-hydroxyinitrile glycosides and belong to plant secondary metabolites (natural products). They are plant constituents derived from amino acids, present in more than 2500 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, headache, loss of consciousness and convulsions with paralysis of the respiratory center of the brain. The lethal dose of hydrocyanic acid for an adult male is 50 to 60 mg/kg body weight [45]. Reducing the level of hydrocyanic acid in plants before use is necessary and this action involves treatments. During flour fermentation, a significant decrease ($p< 0.05$) in hydrocyanic acid content was observed. This result is similar to that of [31] who observed a reduction in hydrocyanic acid content in cooked bean seeds fermented with *Bacillus subtilis* compared to cooked unfermented seeds.

Minerals are essential for the proper functioning of the body. During bean fermentation, an increase in sodium content was observed. This result is similar to that of [46] who observed an increase in sodium content during the fermentation of *Parkia biglobosa* seeds for the production of iru. Although the sodium content was increased during fermentation, it 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 would be due to the use of these by microorganisms for their metabolism. Furthermore, during fermentation, the reduction of antinutritional factors would lead to an increase in the bioavailability of magnesium. Although the magnesium content has been reduced, fermentation makes *Phaseolus lunatus* a good source of magnesium with a daily intake of 400 mg/day [49]. Phosphorus is an essential macromineral that is involved in the structure of bones, teeth, DNA, RNA and the plasma membrane of cells. During fermentation, an increase was observed after 48 hours of fermentation. [46] also observed an increase in phosphorus content during fermentation of *Parkia biglobosa* seeds. Thus, fermentation is beneficial for the phosphorus intake of *Phaseolus lunatus*, the needs of which 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 fluid balance as the most abundant intracellular cation [51]. During fermentation of *Phaseolus lunatus* (L.) flour by *Bacillus subtilis*, an increase in potassium content was observed. This result is similar to that of [46] who also observed an increase in the potassium content of *Parkia biglobosa* seeds during their fermentation. Fermented flour of *Phaseolus lunatus* (L.) with *Bacillus subtilis* constitutes 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 and the body's physiological mechanisms [52]. The calcium content increased during fermentation. Furthermore, the significant reduction of antinutritional factors could lead to an improvement in bioavailability, thus increasing the calcium intake of *Phaseolus lunatus* (L.). Iron is an essential trace element that plays an essential role in oxygen transport and energy metabolism [53]. During bean fermentation, there was a reduction in iron content which was significant only at 48 hours of fermentation. This reduction could be due to the use of iron in the metabolism of microorganisms. Furthermore, this reduction in the iron content during fermentation does not have a considerable influence on the iron intake of *Phaseolus lunatus*

(L.) In fact, the admissible daily intake is 8 to 18 mg/ day for adults [54] and the reduction of antinutritional 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 bean remained constant. The daily zinc intake is 10 mg. *Phasoelus lunatus* (L.) seeds are not good sources of zinc and fermentation does not influence their zinc content. Copper is an essential trace element that acts as a component of many proteins, including many important enzymes [56]. Like zinc, the copper content did not vary during fermentation. Fermented *phaseolus lunatus* (L.) flour could be a good source of copper, the daily intake for an adult is estimated at 1 mg [57].

Antioxidant activity correlates with the occurrence of phytochemicals, including 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 [59] who observed an increase in the polyphenol content of rice bran fermented by *Bacillus sutilis* compared to unfermented bran. [60] also showed that the total polyphenol content of black soybean extract fermented with *Bacillus subtilis* BCRC 14715 is significantly higher ($p< 0.05$) than the respective extract of unfermented black soybean. The increase in polyphenol content in fermented flours could be due to the bioconversion of conjugated forms of phenolic acids into simple forms by the action of β-glucosidase produced by Bacillus subtilis. Indeed, the enzyme β-glucosidase (β-D-glucoside glucohydrolase) catalyzes 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 hydrolyzing glycosides to release free phenolic acids [62].

An increase in the scavenging potential of the DPPH radical and hydrogen peroxide was observed during the fermentation of *P. lunatus* by *Bacillus subtilis.* [63] reported that the DPPH radical inhibition potential of four soybean cultivars fermented with *Bacillus subtilis* for cheonggkjang production was higher than that of unfermented soybean. [64] also reported that naturally fermented and *Bacillus subtilis* fermented soybean for the production of ' ' thuanao' ' has a higher DPPH radical inhibition power than that of unfermented soybean. The high trapping potentials of the DPPH radical and hydrogen peroxide are strongly correlated with the evolution of the contents of total polyphenols and total flavonoids during the controlled fermentation. This strong correlation would reflect the involvement of these phenolic compounds on the antioxidant power of fermented flour.

5. CONCLUSION

Bacillus subtilis is a species of microorganisms used in the production of certain condiments of Asian and African origin. The use of *Bacillus subtilis* in the controlled fermentation of beans (*Phaseolus lunatus*) has made it possible to improve its nutritional quality. Indeed, antinutritional factors such as phytates, tannins, oxalate and hydrocyanic acid have decreased considerably, thus increasing the bioaccessibility of nutrients. Furthermore, the levels of nutrients such as proteins, fiber, reducing sugars, calcium, sodium, potassium and phosphorus have increased. Those of carbohydrates, lipids, ash, total sugars, magnesium and iron have decreased. Does the increased protein content make the fermented bean a good source of protein; which could make it possible to make *Phaseolus lunatus* (L.) flour fermented with *Bacillus subtilis*, an ingredient whose consumption would reduce the problems of protein-energy malnutrition and would increase its interest. Furthermore, the fermentation of flour by *Bacillus subtillis* increases the content of bioactive phenolic compounds and the antioxidant potential by increasing the trapping potential of the DPPH

radical and hydrogen peroxide. Thus, fermentation with *Bacillusubtillis* increases the biological quality of the bean.

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