MICROBIAL FERMENTATION OF *GLYCIN MAX*

Utilization of *Glycine max*: comparing bacterial and fungal fermentation

ABSTRACT

Aims: A comparative study of fungi and bacteria fermentation of soybean (*Glycine max*) was carried out to determine the effect of fermentation on the nutritional composition of their fermented products: tempeh and 'soy-iru'.

Study design: The experiment was carried out in the Department of Microbiology, Ekiti State University, Ado Ekiti, Nigeria, between August, 2017 and July 2018.

Methodology: Soybean was processed into 'soy-iru' (bacterial fermentation) and tempeh (fungal fermentation) and the microbial load, physico-chemical properties, proximate composition, levels of anti-nutritional components (trypsin inhibitor and phytic acid), anti-oxidants (total phenol, total flavonoid and DPPH), in-vitro protein digestibility and vitamins (A, B, C, D, and E) were analyzed.

Result: The microbial load, pH increased progressively during fermentation, while there was a decrease in the titratable acidity (TTA) of the two products. The protein, ash and fat contents of the *Glycine max* cotyledons increased from 29.56, 1.86 and 24.36 in unfermented substrate to 33.61, 2.21 and 26.90 respectively, after 36hrs of fermentation to produce tempeh; however, there was a reduction in crude fibre and carbohydrate content from 2.94 and 41.29 in unfermented substrate to 2.53 and 32.57 respectively, after 36hrs of fermentation. Similar trends were observed during the production of 'soy-iru', however the change in proximate composition was not as significant as observed in tempeh. There was significant decrease in the trypsin

inhibitor and phytic acid levels of the two products. The levels of anti-oxidants, vitamins B, D, E and protein digestibility increased significantly, in both bacterial and fungal-fermented products.

Conclsion: This research has therefore shown that fungal fermentation of *Glycine max* seeds into tempeh may be a better alternative to 'soy-iru' which was obtained from bacterial fermentatoin, because of the significant lower level anti-nutritional factors in the former.

Key word: Glycine max, vitamins, tempeh, soy-iru, anti-nutritional factors

INTRODUCTION

Soybean (*Glycine max*) is a plant legume, known for more than 3000 years in Southeastern Asia [1]. Soybean is one of the widely consumed foods in the world due to its high nutritional value and low cost [2]. It is a legume that has high level of protein, appreciable amount of minerals, vitamins and fibres, some amount of antioxidants, small amounts of saturated fat and absence of cholesterol [2].

Some of the health benefits of soybean include: improved metabolic activities, healthy weight gain, prevention of cancer, boost heart health, relieves menopausal symptoms, boost digestion and improve bone health. However, raw soybean is toxic to non-ruminants due to high concentration of anti-nutritional factors such as trypsin inhibitors and high level of phytic acids. [3]. Most of these anti-nutritional factors present in the raw seeds chelate some important vitamins and minerals, thereby preventing their absorption into the body. Due to the high level of anti-nutritional factors, processing is required before the seeds can be consumed by non-ruminant, since the goal of eating is to get adequate amount of nutrients in the diet [4]. Fermentation is one of the processing methods that can be employed in the processing of soybean into soyiru. Bacterial fermentation (using *Bacillus subtilis* strains) lead to production of 'soy-iru', natto, thua-nao; while fungal fermentation (using *Rhizopus oligosporium*) lead to

production of tempeh [5]. This research aims at comparing the bacterial fermented product ('soy-iru') of soybean, with the fungal fermented product (tempeh), on the bases of nutritional factors, anti-nutritional factors and anti-oxidant levels.

Materials and Methods

Sources of Materials:

The *Glycine max* seeds were purchased from Oja Oba in Ado-Ekiti. The pure cultures of *Bacillus subtilis* strains and *Rhizopus oligosporium* were obtained from the stock cultures kept in the Laboratory of Department of Microbiology, Ekiti State University, Ado-Ekiti, Nigeria.

Processing of the seed

The method described by [4] on the production of 'soy-iru' from soybean *(Glycine max)* seeds was adopted. Five hundred grams (500g) of soybean seeds were sorted, washed andz boiled for 2h. The boiled seeds were dehulled to remove the seed coat, washed and boiled again for 1 hour. The water was drained off and the beans were fermented in an incubator at 35^{o}C for 36h. Samples were taken at every 12h and analyzed for microbial load, physico-chemical properties, proximate, anti-nutritional content, antioxidant level, vitamin content and protein digestibility.

Preparation of spores' suspension for 'tempeh' production

The procedure described by [5] was adopted to prepare spore suspension. Five grams (5g) of Malt Extract Agar (MEA, Oxoid) was weighed and dissolved in 100ml of distilled water in a 250ml conical flask. The medium was homogenized and sterilized in an autoclave at 121^{o}C for 15 minutes. One gram of bacteriological peptone (Lab M) was weighed and dissolved in 100ml of distilled water in a 250ml conical flask. This was also sterilized by autoclaving. The sterile

MEA was poured into sterile plates and allowed to solidify. One gram of *Rhizopus oligosporus* NRRL 2710 powder was added aseptically into 5ml sterile peptone water in a 100ml conical flask and it was mixed together to disperse the powdered inoculum. One millilitre (1ml) was inoculated into the MEA plate. The agar plates were inverted and incubated at 30^{o}C for 72h. After incubation, the spores were harvested by pouring 5ml sterile peptone water into each of the sporulated culture in the Petridishes and scrapped, using wire loop. The harvested culture was filtered through sterile non-absorbent cotton wool into a sterile conical flask to obtain the spores' suspension.

Laboratory production of 'tempeh' from soybean *(Glycine max)* seeds

'Tempeh' was prepared by fermenting soybean according to the procedure of [5]. The soybeans (*Glycine max)* were washed and boiled partially for 30 mins. The soybeans were dehulled, cleaned and soaked in clean water overnight. The soaked soybeans were then boiled for 45 mins. The moist cotyledons were drained properly air-dried and cooled for 20-30 minutes after which they were inoculated with spores' suspension of *Rhizopus oligosporus* NRRL 2710 with ratio 1:50 (v/w). The cotyledons were lightly packed into sterile perforated baking tins covered with perforated aluminum foil paper and incubated for 24 h at 35^{o}C. Samples were taken at every 12h and analyzed for microbial load, physico-chemical properties, proximate, anti-nutritional content, antioxidant level, vitamin content and protein digestibility.

Microbiological analysis: The microbial load (viable counts) was determined using serial dilution and plating technique on nutrient agar (NA) plates. The bacterial isolates were partially characterized on the bases of cultural, morphological and biochemical properties [6].

pH determination:

Five grams (5g) of each sample was homogenized and mixed with 100 ml of distilled water. The pH of each homogenate was determined with a Pye Unicam pH meter (Model PW9409). The determination was carried out in triplicates.

Total titratable acidity determination:

The suspension from the pH determination was filtered and 20 ml of the filtrate was titrated against 0.1M NaOH using 1 drop of phenolphthalein as indicator [7].

Moisture content determination:

Five grams (5g) of each sample was weighed separately into pre-weighed aluminum foil. The foil paper and its content was put in oven at 80^{o}C overnight and weighed intermittently until a constant weight was achieved. The new weight was subtracted from the weight of the wet sample. The percentage moisture content was calculated [8].

Proximate analysis:

The proximate compositions of the fermented and unfermented samples were determined using standard procedures of [8]. The parameters determined were protein, ash, crude fibre, fat and carbohydrate.

Determination of Anti-nutritional Factors

Phytic acid

The method of [9] was employed in the determination of phytic acid. Four grams (4 g) of finely ground sample was soaked in 1 L of 2% HCl inside conical flask for 3h and was filtered. Five milliliters (5 ml) of 0.03%NH4SCN was added as indicator and 50 ml of distilled water also added. This was titrated against ferric chloride solution which contained 0.05 mg of iron (Fe) per ml of FeCl3. The iron equivalent was obtained and the phytate content in mg/100 mg of dried sample was calculated.

Trypsin inhibitor

The trypsin inhibitor activity (TIA) in the sample was determined according to the method of [10]. The digest contained 1.0 g of the sample, 40 μg of trypsin and 2 mg of Nalpha-benzoyl-DL-Arginine-Pnitroanilidehydrochloride. The absorbance was read at 410 nm.

Determination of Anti-oxidants

Total phenol

The total phenol contents of the samples were determined using the method reported by [11, while total flavonoids content and 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical-scavenging ability of the samples were determined by the method of [12] and [13], respectively.

Determination of Vitamins

Vitamin A was determined by the method of [14]; vitamin B by the method of [15] vitamin C by the method of [16], while vitamins D and E were determined by the methods of Pearson [14]

Determination of multi-enzyme *In vitro* Protein Digestibility

The method of Singh and Krikorian [17] was adopted in the determination of multi-enzyme in-vitro protein digestibility of the samples, using procaine pancreatic trypsin as enzyme. The absorbance was read at 700 nm against reagent blank. The standard calibration (STD) curve was prepared using 100 μg/ml of Bovine Serum Albumen (BSA).

Results

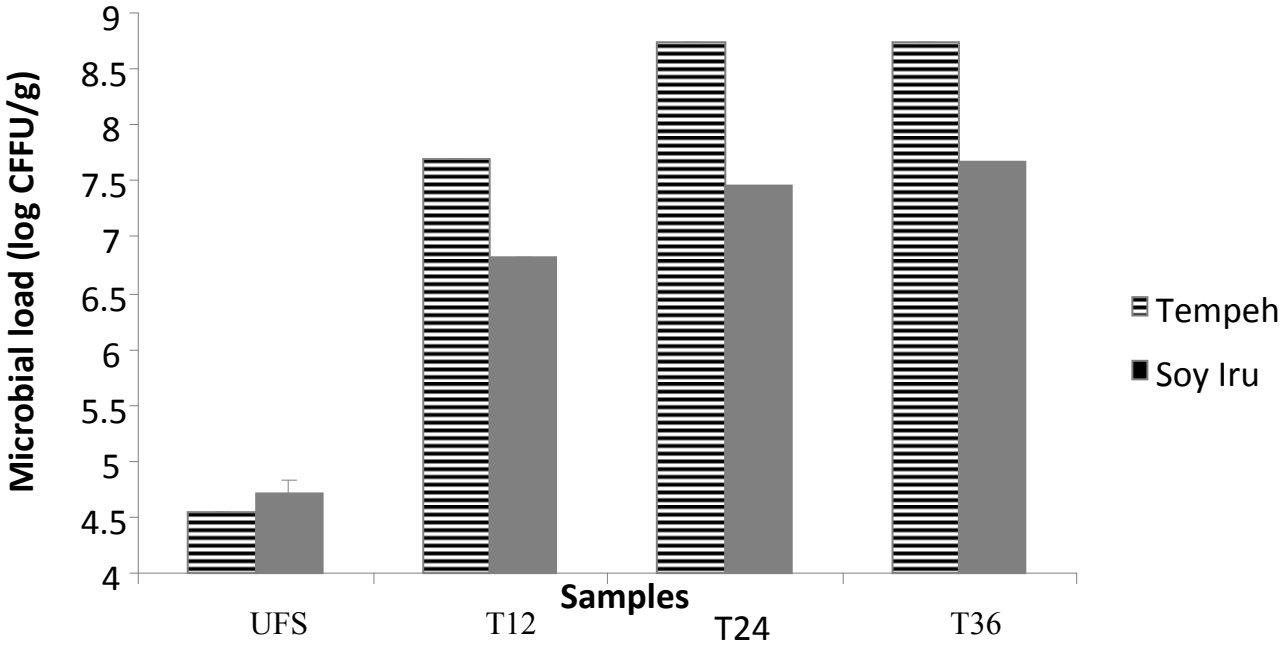
Figure 1 shows the microbial load of the samples during fermentation of *Glycine max* to 'tempeh' and 'soy-iru', respectively. The microbial load increased progressively at different periods of fermentation, from 4.55 log CFU/g to 8.74 log CFU/g ('tempeh') and 7.67 log CFU/g ('soyiru'), respectively. The pH of the substrate increased significantly during the fermentation (Fig 2) from 5.50 to 6.94 (tempeh) and 5.50 to 8.079 ('soyiru'). The total titratable acidity (TTA)

(Fig 3) of *Glycine max* reduced from 3.09×10^{-2}N to 2.17×10^{-2}N (tempeh) and from 2.57×10^{-2}N to 1.10×10^{-2} ('soy-iru'). As shown in Figure 4, the moisture content of the substrate decreased from 20.3% to 16.53% in tempeh; but increased from 45.33% to 59% in 'soyiru'.

The proximate compositions of 'tempeh' and 'soy-iru' during fermentation are shown in

Tables 1. The protein content of the *Glycine max* contyledons increased from 29.56% to 33.67% during fermentation of tempeh. There were also increases in the ash and fat contents. However, the crude fibre and carbohydrate content decreased from 2.94% to 2.53% and 41.29% to 32.57%, respectively. Similar trends in the values of the parameters assessed were observed during 'soy-iru' fermentations.

Table 2 shows the anti-nutritional factors and the anti-oxidants level of the fermenting substrate and products. The trypsin inhibitor level decreased significantly from 55.84mg/g to 44.33mg/g (tempeh) and from 64.35mg/g to 45.02mg/g ('soy iru'), respectively. Similarly, phytic acid content decreased significantly from 38.45mg/g to 8.43mg/g and 55.76 to 9.89 in 'tempeh' and 'soy iru' respectively after fermentation. There was significant



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157 **Figure 1: Microbial load (log CFU/g) of 'tempeh' and 'soy-iru' during fermentation of *Glycine max* seeds**

158 **Key: UFS= unfermented substrate, T12= sample at 12h of fermentation boiling, T24 = sample at 24h of**

159 **fermentation, T36= sample at 36h of fermentation.**

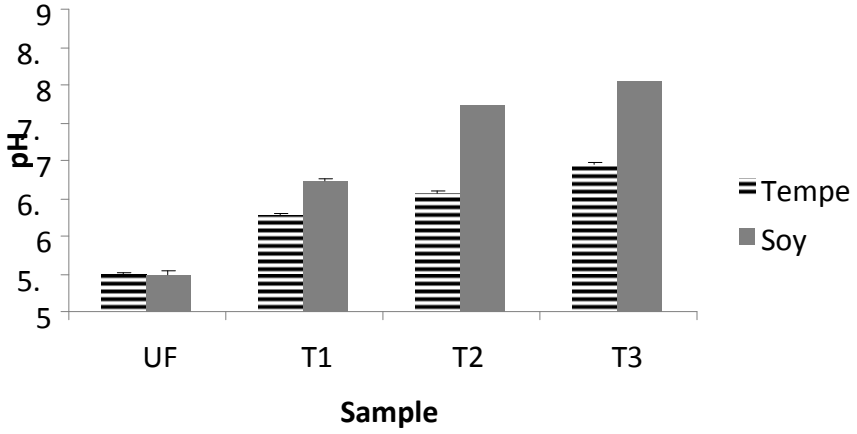

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Figure 2: pH of 'tempeh' and 'soy-iru' during fermentation *Glycine max* seeds

Key: UFS= unfermented substrate, T12= sample at 12h of fermentation boiling, T24 = sample at 24h of fermentation, T36= sample at 36h of fermentation.

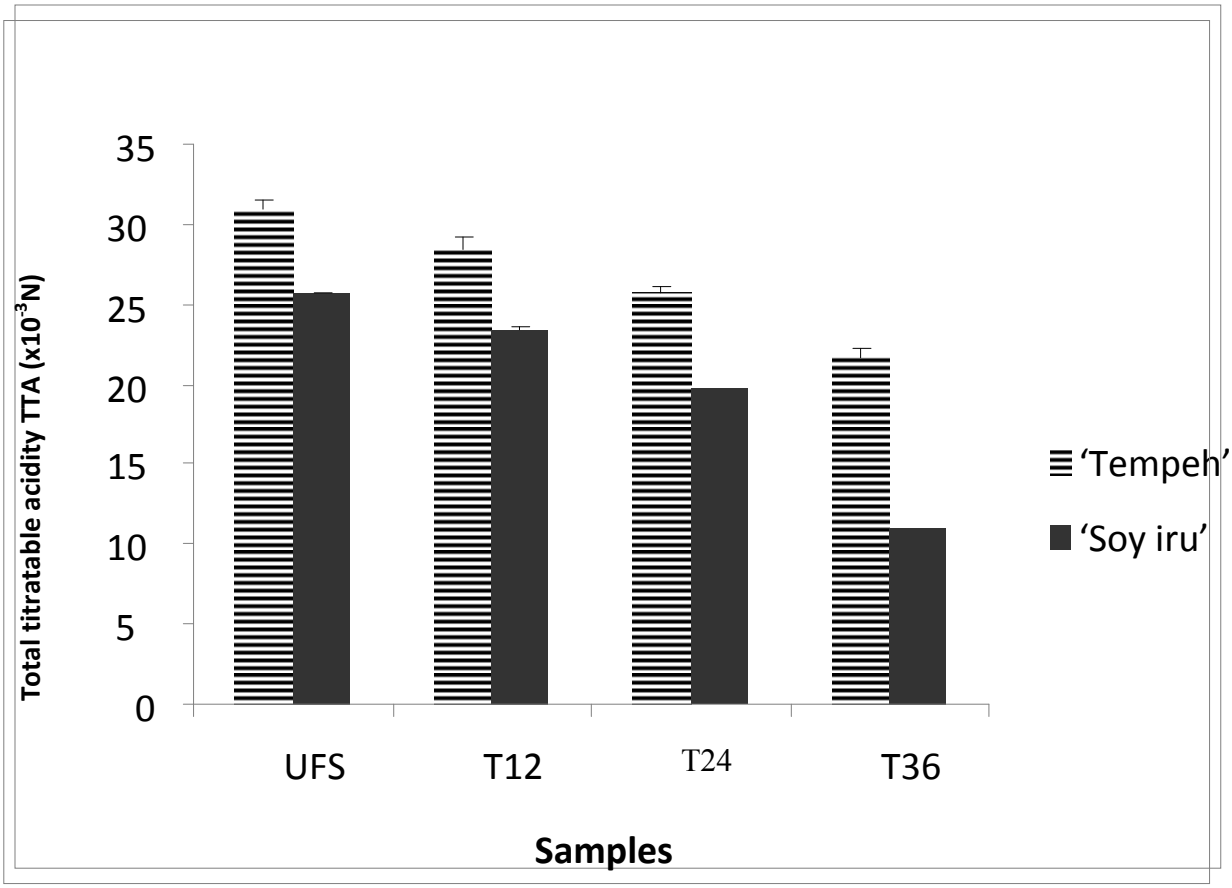
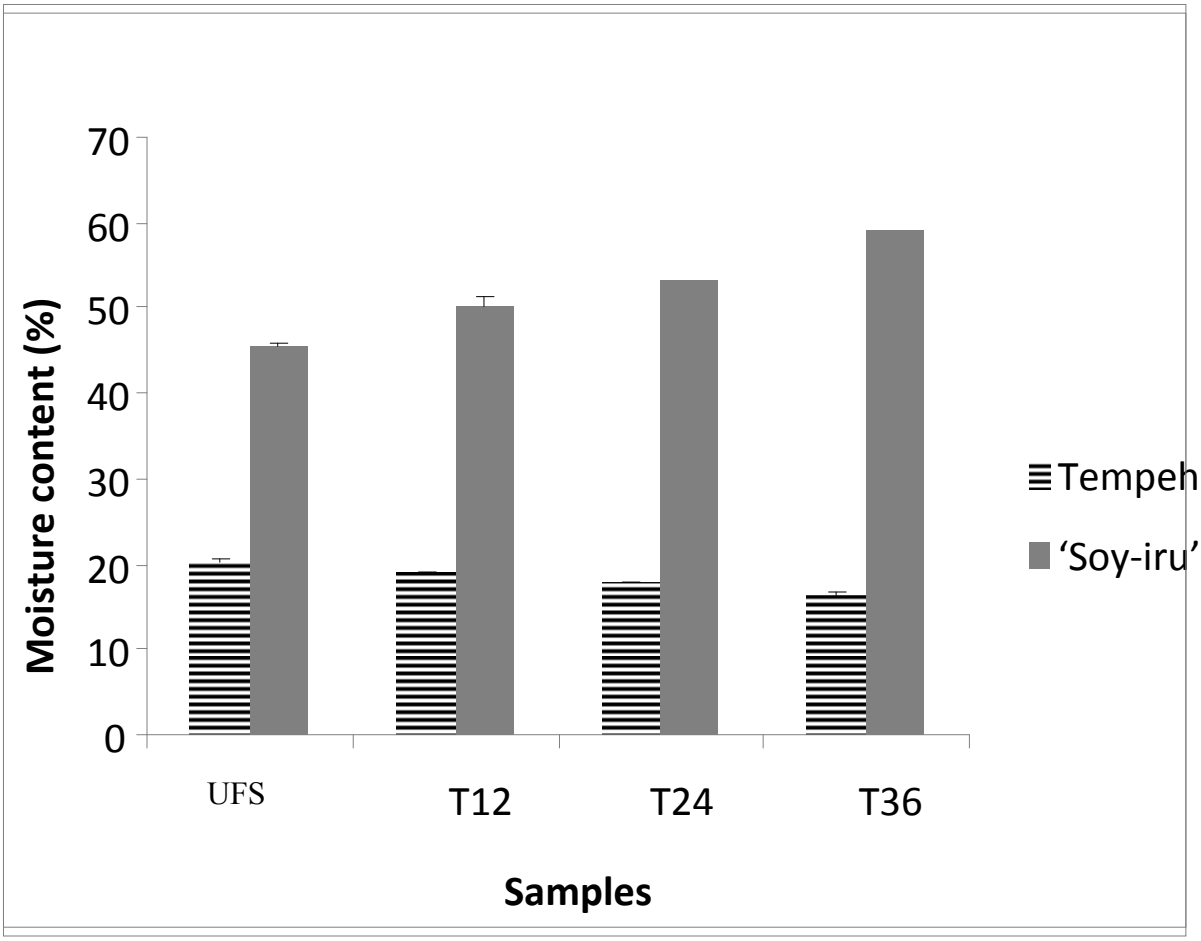


Figure 3: Total titratable acidity (TTA) 'tempeh' and 'soy-iru' during fermentation of *Glycine max* seeds

Key: UFS= unfermented substrate, T12= sample at 12h of fermentation boiling, T24 = sample at 24h of fermentation, T36= sample at 36h of fermentation.

fermentation, T36= sample at 36h of fermentation.



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193 **Figure 4: Moisture content (%) of 'tempeh' and 'soy-iru' during fermentation of *Glycine max* seeds**

194 **Key: UFS= unfermented substrate, T12= sample at 12h of fermentation boiling, T24 = sample at 24h of**

195 **fermentation, T36= sample at 36h of fermentation.**

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198 Table 1: Proximate composition (%) of 'tempeh' and soy-iru during fermentation of *Glycine max*

199 seeds

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SAMPLES	PROXIMATE CONMPOSITION (%)									
	PROTEIN		ASH		FIBRE		FAT		CARBOHYDRATE	
	Tempeh	Soy-iru	Tempeh	Soy-iru	Tempeh	Soy-iru	Tempeh	Soy-iru	Tempeh	Soy-iru
UFS	29.56^{c} ±0.48	24.51^{d} ±0.01	1.86^{d} ±0.01	0.98^{d}± 0.01	2.94^{a}± 0.01	3.86^{a}± 0.10	24.36^{c} ±0.02	20.06^{b} ±0.10	41.29^{a} ±0.44	50.12^{a}± 1.17
T12	31.79^{b} ±0.23	26.97^{c} ±0.02	1.98^{c} ±0.01	1.34^{c}± 0.00	2.84^{b}± 0.01	3.78^{a}± 0.17	25.24^{b} ±0.09	21.97^{c} ±0.01	38.16^{b} ±0.31	46.26^{b}± 0.47
T24	31.24^{b} ±0.45	29.00^{b} ±0.58	2.13^{b} ±0.02	1.53^{b}± 0.05	2.63^{c}± 0.00	3.61^{b}± 0.06	26.85^{a} ±0.02	23.60^{b} ±0.13	34.79^{c} ±0.50	42.36^{c}± 0.00
T36	33.61^{a} ±0.00	31.27^{a} ±0.06	2.21^{a} ±0.02	1.74^{a}± 0.04	2.53^{d}± 0.02	3.46^{b}± 0.06	26.90^{a} ±0.10	32.57^{a} ±0.04	32.57^{d} ±0.04	38.73^{d}± 0.21

201 **Key: UFS= unfermented substrate, T12= sample at 12h of fermentation boiling, T24 = sample at 24h of**
202 **fermentation, T36= sample at 36h of fermentation. Values that have superscript in a column are not**
203 **significantly different at P = 0.05.**

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207 Table 2: Anti-nutritional factors (mg/g) and antioxidant levels (mg/g) of 'tempeh' and soy-iru
208 during fermentation of *Glycine max* seeds

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SAMPLES	Antinutritional factors (mg/g)				Antioxidants (mg/g)					
	Trypsin inhibitor		Phytic acid		Total phenol		Total flavonoids		Free radical scavengers	
	Tempeh	Soy-iru	Tempeh	Soy-iru	Tempeh	Soy-iru	Tempeh	Soy-iru	Tempeh	Soy-iru
UFS	55.84^{a} ±0.29	64.35^{a} ±0.28	38.45^{a} ±0.48	55.76^{a} ±0.47	0.23^{d} ±0.01	0.46^{d}± 0.00	0.04^{d}± 0.00	0.03^{c}± 0.03	66.45^{d} ±0.65	68.82^{d}± 1.00
T12	51.11^{b} ±0.07	52.43^{b} ±0.00	19.23^{b} ±0.95	29.36^{b} ±0.47	0.43^{c} ±0.00	0.49^{c}± 0.00	0.07^{c}± 0.00	0.08^{b}± 0.00	73.33^{c} ±0.75	74.47^{c}± 1.00
T24	46.29^{c} ±0.00	49.36^{c} ±0.00	13.71^{c} ±0.00	17.30^{c} ±0.00	0.49^{b} ±0.00	0.56^{b}± 0.01	0.09^{b}± 0.00	0.10^{b}± 0.01	75.91^{b} ±0.38	85.16^{b}± 1.30
T36	44.33^{d} ±0.14	45.02^{d} ±0.00	8.43^{d}± 0.00	9.89^{d}± 0.00	0.62^{a} ±0.00	0.63^{a}± 0.01	0.15^{a}± 0.01	0.21^{a}± 0.02	86.45^{a} ±0.00	88.17^{a}± 1.34

210 **Key: UFS= unfermented substrate, T12= sample at 12h of fermentation boiling, T24 = sample at 24h of**
211 **fermentation, T36= sample at 36h of fermentation. Values that have superscript in a column are not**
212 **significantly different at P = 0.05.**

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215 Table 3: Vitamins (mg/g) and protein digestibility levels (%) of tempeh and 'soy-iru' during
216 fermentation of *Glycine max* seeds

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SAMPLES	Vitamins (mg/g)										Protein digestibility (%)	
	A		B		C		D		E			
	Tempeh x10^2	Soy-iru x10^2	Tempeh	Soy-iru	Tempeh	Soy-iru	Tempeh	Soy-iru	Tempeh	Soy-iru	Tempeh	Soy-iru
UFS	10.73^{a} ±0.69	$14.95.^{a}$ ±0.23	0.26^{d}± 0.00	0.15^{d}± 0.00	0.18^{a}± 0.02	0.46^{a}± 0.02	0.18^{d}± 0.00	0.41^{d}± 0.00	0.28^{d}± 0.03	0.46^{d}± 0.00	28.78^{d} ±0.13	32.96^{d} ±0.21
T12	5.81^{b}± 1.10	8.62^{b}± 0.04	0.41^{c}± 0.00	0.41^{c}± 0.00	0.14^{b}± 0.01	0.23^{b}± 0.00	0.46^{c}± 0.02	0.49^{c}± 0.06	0.58^{c}± 0.00	0.58^{c}± 0.00	46.10^{c} ±0.10	40.23^{c} ±0.20
T24	4.43^{c}± 0.00	5.34^{c}± 0.37	0.56^{b}± 0.00	0.56^{b}± 0.00	0.12^{c}± 0.00	0.13^{c}± 0.01	1.28^{b}± 0.02	1.40^{b}± 0.00	1.14^{b}± 0.03	1.14^{b}± 0.03	54.90^{b} ±0.10	47.70^{b} ±0.65
T36	3.28^{d}± 0.00	4.17^{d}± 0.00	1.09^{a}± 0.21	1.09^{a}± 0.21	0.10^{d}± 0.01	0.06^{d}± 0.00	1.59^{a}± 0.00	1.84^{a}± 0.00	1.51^{a}± 0.00	1.51^{a}± 0.00	62.02^{a} ±0.02	57.13^{a} ±0.61

220 **Key: UFS= unfermented substrate, T12= sample at 12h of fermentation boiling, T24 = sample at 24h of**
221 **fermentation, T36= sample at 36h of fermentation. Values that have superscript in a column are not**
222 **significantly different at P = 0.05.**

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increase in the anti-oxidants levels of the substrate during fermentation. The total flavonoids contents increase from 0.04mg/g to 0.15mg/g in ‘tempeh’ and 0.03mg/g to 0.21mg/g in ‘soy-iru’. A similar trend was observed in the contents of total phenol and diphenylpycrylthdrazyl (DPPH) radical scavengers during the fermentation.

The vitamins and the in-vitro protein digestibility of the fermenting substrate and fermented products during fermentation of *Glycine max* to tempeh and ‘soy-iru’ are presented in Table 3. The vitamins A and C contents reduced during the fermentation processes. Vitamin A contents reduced from 1072.66mg/g to 327.64mg/g (‘tempeh’) and 1495.33mg/g to 417.24mg/g (‘soy iru’); while vitamin C contents reduced from 0.18mg/g to 0.10mg/g in ‘tempeh’ and 0.46mg/g to 0.06mg/g in ‘soy-iru’. However, vitamins B, D and E increased significantly during the fermentation. There was a significant increase in the in-vitro protein digestibility from 28.78% to 62.02% in tempeh; similar result was observed for ‘soy-iru'.

Discussion

The steady increase in microbial load during the fermentation might be due to availability of nutrients released from the cotyledons by the action of fermentation and the utilization of these nutrients by the fermenting organisms for their metabolic activities. This is in agreement with the previous result gotten by Omodara and Aderibigbe [18] when working on ‘iru’. The increase in the protein, ash, fat and anti-oxidants might be attributed to secretion of hydrolytic enzymes by the fermenting organisms [19]. The decrease in the level of phytic acid and trypsin inhibitor may be attributed to the metabolic activities of the fermenting organism. It may also be due to breaking down of these complexes by the enzymes produced the fermenting organisms [18]. The increase in the Vitamin B, D and E with increase in fermentation might be due to the release of this vitamin from their bond state by the activities of the fermenting organisms while the

decrease in Vitamin A and C might be due to the metabolic activities of the fermenting organisms. It was found that fermentation had a significant increase in in- vitro protein digestibility of the two products. The microorganisms involved in the fermentation produce proteolytic enzymes which degrade complex proteins, hence increase in digestibility [20].

In conclusion fermentation was found to enhance of the nutritional qualities of *Glycine max* seeds when fermented into tempeh (using *Rhizopus oligosporus* NRRL 2710) and 'soyiru' (using *Bacillus subtilis* 3A); as both have significant reduction in the anti-nutritional contents (phytic acid and typsin inhibitor). However, tempeh may be better alternative to process the 'soybean' because of its lower anti-nutritional factors

Conflicts of Interest

The authors declare no conflicts of interest

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