Influence of alkaline fermentation time *on in vitro* nutrient digestibility, bio- & techno-functionality, secondary protein structure and macromolecular morphology of locust bean (*Parkia biglobosa*) flour

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A B S T R A C T

Present investigation aimed at evaluating the variation in anti-nutritional factors, phytochemical constituents, bio-functional and technological attributes of locust bean (*Parkia biglobosa*) flour after alkaline fermentation treatment for different time periods (0, 24, 48 and 72 h). Significant reduction ($p < 0.05$) in phytate (12.59–5.16 mg/g), saponin (6.87–1.92 mg/g) and tannin (2.86–1.03 mg/g) contents was observed linearly with the fermentation time. Furthermore, *in vitro* starch and protein digestibility were found to increase from 5.29 to 24.82 g/100 g and 66.47–74.29 g/100 g respectively in response to treatment time. ATR-FTIR spectrum in Amide II region (1600-1700 cm^{-1}) revealed that β-sheet structure prevailed in raw and fermented flours; however, there was significant increase in the α-helix and random coils. Scanning electron micrographs and X-ray diffractograms presented degradation of granular integrity of starch and three-dimensional protein networking. These compositional and structural changes lowered the hydration and emulsification potential while oil absorption and water solubility were found to enhance from 0.86 to 1.01 g/g and 3.16–10.41% respectively. Pearson's correlation matrix showed significant relationship among anti-nutritional factors and nutrient digestibility while principal component analysis validated the differences among variables and observations through two key components of 75.85 and 22.44% respectively.

1. Introduction

Locust bean (*Parkia biglobosa*) tree belonging to family leguminosae holds prime significance in West African nations due to nutritional and medicinal potential of its seeds, stems, barks, roots and leaves (Burlando et al, 2019). Locust bean seeds are embedded in powdery pulp inside the fruit pods and are generally fermented prior to consumption, often utilized to produce protein rich condiments (Adeloye & Agboola, 2020). However, roasted seeds also make excellent coffee substitute. Further, its leaves and flowers are consumed as vegetables and added to salads while the mucilaginous pulp surrounding the seeds may be consumed fresh or processed to develop flavored beverage (Martin, Campbell, & Ruberté, 1987). Raw seeds constitute moisture content (1.5–13%), carbohydrates (13–54%), protein content (20–36%), fat content (8–32%), fibres (0.4–17%) and minerals (1–6%) of its edible portion (Singh, Rehal, Kaur, & Jyot, 2015) (Singh et al., 2015) (Termote et al.,

2020). They also contain several phytochemical constituents belonging to polyphenols, flavonoids and vitamins which exert different bio-activities to benefit human health.

Fermentation is an essential folk and traditional processing treatment for legume seeds in many parts of the world, predominantly in African nations to enhance the nutritive and organoleptic characteristics of the seeds. On the same note, locust bean seeds are also fermented to produce a famous indigenous condiment, known as *iru/daddawa* in different parts of Central and Western Africa (Falade & Akinrinde, 2020). Fermented locust bean is an important culinary preparation to enhance flavor and meatiness of sauces, soups and other food products. Conventional fermentation process is often based on natural contamination; however, *Bacillus subtilis* are the major bacterial strains involved (Gernah, Inyang, & Ezeora, 2007). Fermentation is accompanied with strong ammoniacal odor due to metabolization of hydrolyzed amino acids by fermentation-induced proteolytic action (Gernah et al., 2007).

Fermentation does not only modulate the sensory properties of the seeds, but significant variation in nutrient composition, structural & molecular interactions have also been reported (Oboh et al, 2008).

Hydrolysis of cell wall to release bound polyphenols and their biosynthesis during fermentation contribute to enhanced antioxidant value of the grains (Adebo & Gabriela Medina-Meza, 2020). Higher antioxidant potential of food reduces the risk of several chronic degenerative diseases by scavenging reactive oxygen species and other free radicals which otherwise result in oxidative stress (Kaur, Sharma, & Singh, 2019). Fermentation of locust beans has been explored to modulate the compositional parameters, mainly protein, carbohydrates, anti-nutritional factors and mineral content (Eka, 1980); however, limited reports have been dedicated to modification in the phytochemical profile and antioxidant potential after treatment. Further, the interactions between phytonutrients and macromolecules during fermentation could affect the bioavailability and digestibility of several constituents and these effects have not been critically examined in earlier investigations. Fermentative processing of locust bean remained limited to household scale despite their nutritive profile and potential health benefits which could be due to lack of optimization and standardization of the process. Moreso, changes in the protein secondary structure and morphology during fermentation remained to be investigated thoroughly. Thereby, present article was aimed to observe the impact of fermentation time on bioactive profile and techno-functional characteristics and to understand the molecular interactions and protein secondary structure as influenced by alkaline fermentation process.

2. Material and methods

2.1. Raw material and processing

Locust bean (*Parkia biglobosa*) seeds were procured from local market of Ludhiana, India. Seeds (~250 g each independent batch) were thoroughly washed with distilled water and cooked in boiling water (1:5 seed to water ratio) for 90 min with added potash (1% potassium bicarbonate) to maintain alkaline environment and left overnight. Seeds were then added with ash and pounded until husk layers was completely removed. Cotyledons were then exposed to natural fermentation for different intervals (0, 24, 48, and 72 h) at 38 °C in a temperature-humidity control chamber (SCS Equipments, India). Seeds were then dried in a tray drier at 45–50 °C to obtain moisture content of approximately 8 percent. Raw locust bean seeds, after removal of husk layers, were considered as control. Seeds were milled using cyclotec milling machine (Newport Scientific, Australia) to obtain flour of uniform particle size of 250 μm.

2.2. Anti-nutritional factors

Anti-nutritional constituents were estimated in terms of tannin content, phytate content and saponins. Tannins were estimated using Folin-Denis reagent and expressed as mg tannic acid per g dry locust bean flour (LBF) (Sharma, Kaur, & Kaur, 2017). Tannins were extracted from 500 mg flour in 25 mL of 10% methanol using reflux for 1–2 h 250 μL of extract was added with 1.25 mL Folin-Denis reagent and 2.5 mL saturated sodium carbonate. Final volume of 25 mL was made with deionized water and the contents were incubated at 25 °C for 30 min. Absorbance was then read at 700 nm. Tannic acid (25–125 μg) was taken as standard for expressing the results. Phytate content was measured in terms of mg phytic acid per g of LBF on dry basis using

method adopted by Sharma et al. (2017). For extraction, 500 mg LBFs were added with 20 mL nitric acid (0.5 M) and shaken for 4 h and final volume was made to 25 mL for further estimation. 500 μL of extract was added to 0.9 mL deionized water followed by 1 mL ammonium ferric sulphate solution. Contents were mixed well and held at 100 °C for 20 min before addition of 5 mL amyl alcohol. Thereafter, 100 μL of 10% ammonium thiocyanate solution was added and incubated for 15 min at 25 °C followed by centrifugation for 10 min at 1000 g. Absorbance was read at 465 nm. Standard sodium phytate salt was taken in the range of 40–200 μg for estimation. Saponins were estimated in terms of mg saponin per g of dry LBF (Baccou et al, 1977). Fat free methanolic extract (1 mL) was kept in boiling water bath to remove excess of methanol and added with 2 mL ethyl acetate followed by 1 mL of working reagent (0.5 mL anisaldehyde in 99.5 mL ethyl acetate). Further, 1 mL of concentrated sulphuric acid was added, and absorbance was noted at 430 nm exactly after 10 min. Saponin extra-pure was taken as standard for estimation in the range of 20–100 μg.

2.3. Bioactive constituents

Phytochemical compounds were estimated in terms of total phenolic content and total flavonoid content using methods adopted by Sharma, Dar, Sharma, and Singh (2021). Extraction was carried out in 80% acidified methanol (0.1%) using two cycles of reflux of 120 min each. For estimation of total phenolic content, 500 μL of extract was added with 500 μL of aqueous methanol and 5 mL freshly prepared Folin-Ciocalteu reagent (10 times diluted with deionized water). Thereafter, 4 mL of 7.5% sodium bicarbonate solution was added and reaction mixture was incubated at 37 °C for 2 h. Absorbance was then recorded at 725 nm and results were expressed in terms of mg gallic acid equivalent (GAE) per 100 g dry LBF. Total flavonoid content was measured using aluminium chloride-potassium acetate assay. Methanolic extract (2 mL) was added with 10 μL of 10% AlCl$_3$, 100 μL of 1 M potassium acetate and 2.8 mL of deionized water and the contents were then incubated at 25 °C for 30 min. Absorbance was recorded at 415 nm and results were expressed as mg quercetin equivalent (QE) per 100 g of dry LBF.

2.4. Antioxidant potential

Methanolic extracts were investigated for anti-oxidant activity of raw and fermented LBF in terms of DPPH· radical scavenging activity (RSA), ABTS·+ RSA and metal chelation (Fe^{+2}). For DPPH· assay, 100 μL of methanolic extract was added with 3.9 mL of 2 mM DPPH solution prepared in absolute methanol and after exactly 30 min, absorbance was noted at 515 nm using methanol as blank (Brand-Williams, Cuvelier, & Berset, 1995). Percent (%) RSA was calculated using equation (1). ABTS·+ RSA was observed by adding 5 mL ABTS working solution to 1 mL of sample methanolic extract. The absorbance of reaction mixture was recorded at 734 nm exactly after 6 min of incubation at 30 °C and the results were expressed as μM Trolox equivalent (TE) per g dry LBF. Metal chelation was estimated by ferrous chloride-ferrozine assay (Yilmaz, Brandolini, & Hidalgo, 2015); 500 μL of methanolic extract was added with 50 μL of 2 mM FeCl$_2$ and 1.8 mL of absolute methanol. Thereafter, 100 μL of 5 mM ferrozine solution was added to the reaction mixture and absorbance was noted at 562 nm exactly after 10 min. Metal chelation (Fe^{+2}) was calculated using equation (2) (Sharma, Gujral, & Singh, 2012).

$$\% \ RSA = \frac{(Absorbance \ at \ 0 \ minutes \ - \ absorbance \ at \ 30 \ minutes)^* \ 100}{Absorbance \ at \ 0 \ minutes} \tag{1}$$

$$\% \ Metal \ chelation \ (Fe^{+2}) = \frac{(Absorbance \ of \ control \ - \ absorbance \ of \ sample)*100}{Absorbance \ of \ control} \tag{2}$$

2.5. In vitro starch and protein digestibility

In vitro starch digestibility (IVSD) was measured after enzymatic hydrolysis using α-amylase in terms of g maltose released per 100 g LBF after 2 h (Sharma et al., 2021). Maltose standard was taken in the range of 200–1000 µg 100 mg flour was added with 100 mg α-amylase and 20 mL phosphate buffer (pH-6.9). Incubation was done at 37 °C in a shaking water bath for 120 min and thereafter contents were filtered. 1 mL of extract was added to 2 mL dinitro-salicylic acid and exposed to boiling water bath for 5 min to terminate the reaction. Volume was made to 50 mL using deionized water and absorbance was read at 540 nm. Maltose was used as standard. *In vitro* protein digestibility (IVPD) was estimated using pepsin-pancreatin digestion (Sharma et al., 2021). 500 mg flour was digested with pepsin solution (pH-1.9) for 120 min at 37 °C using shaking water bath followed by 120 min of pancreatin digestion under similar condition at pH- 8.0. Contents were centrifuged at 4900 g for 15 min and the supernatant was discarded. Residue was tested for protein content and IVPD was calculated as % difference in undigested and hydrolyzed flour samples.

2.6. Color profile

Tristimulus color parameters i.e. L*, a* and b* were determined using Hunter Lab colorimeter (MiniScan XE Plus, Reston, USA). Furthermore, tristimulus values were used to calculate hue, chroma and overall color difference (ΔE) with following equations (3)–(5) as adopted by Sharma, Dar, Sharma, and Singh (2021).

$$Hue = arctan\frac{b}{a} \tag{3}$$

$$Chroma = \sqrt{a^2 + b^2} \tag{4}$$

$$\Delta E = \sqrt{\Delta L^2 + \Delta a^2 + \Delta b^2} \tag{5}$$

2.7. Techno-functional properties

2.7.1. Water absorption capacity (WAC) and water solubility index (WSI)

WAC and WSI were assessed by hydrating ~2.0 g of flours with 20 mL deionized water followed by continuous shaking for 30 min. Contents were then centrifuged at 4900 g for 10 min (Sharma et al., 2021). Supernatants were taken in pre-weighed Petri-dishes and kept at 100 °C until completely dried. Weight of residual gel pellets were recorded to calculate WAC using formulae given in equation (6). WSI was determined by recording the weight of solids in the supernatants and calculations were done using equation (7).

$$WAC \ (g/g) = \frac{Weight \ of \ gel \ pellet \ (g)}{Weight \ of \ sample \ (g)} \tag{6}$$

$$WSI \ (\%) = \frac{Weight \ of \ solids \ in \ supernatant \ (g)^* \ 100}{Weight \ of \ sample \ (g)} \tag{7}$$

2.7.2. Oil absorption capacity (OAC)

OAC was determined with ~2.0 g of flour added with 20 mL vegetable oil exposed to continuous shaking for 30 min. Contents were then centrifuged at 4900 g for 10 min and supernatants were discarded. Ratios of weight of gel pellet to weight of sample were taken as OAC and expressed in terms of g/g (Singh, Sharma, & Singh, 2017a).

2.7.3. Swelling power (SP)

Exactly 500 mg flour was suspended in 15 mL distilled water and slurry was held in boiling water bath for 30 min with repeated shaking after every 5 min. Contents were then cooled to room temperature followed by centrifugation at 4900*g* for 10 min. Supernatant was discarded and ratio of weight of swollen gel pellet to weight of sample was noted as SP expressed in terms of g/g (Singh, Sharma, & Singh, 2017b).

2.7.4. Gel consistency (GC)

GC was assessed in acidic environment with exactly 200 mg of LBF added with 0.2 mL ethanol (95%) and 3 mL acetic acid (0.1 M). The suspension was then held in boiling water bath for 10 min and cooled to room temperature. Tubes were then laid on levelled surface for 60 min to note the migration of the gel in terms of nearest millimeter (Singh et al., 2017b).

2.7.5. Emulsion activity (EA) and emulsion stability (ES)

The method adopted by Singh et al. (2017a) was undertaken for the assessment of emulsification properties. Locust bean flour (2.0 g) was taken in graduated tube and added with 20 mL each of cold distilled water and refined vegetable oil. The contents were then shaken gently for 20 min followed by centrifugation at 4900*g* for 10 min. Tubes were carefully taken out and EA was estimated by using equation (8). Further, for ES, tubes were exposed to 80 °C water bath for 30 min followed by cooling to room temperature and centrifugation was repeated. ES was calculated using equation (9).

$$EA \ (\%) = \frac{(Height \ of \ emulsion \ layer) \ *100}{Height \ of \ whole \ layer} \tag{8}$$

$$ES \ (\%) = \frac{(Height \ of \ emulsion \ layer \ after \ heat \ treatment) \ *100}{Height \ of \ emulsion \ layer \ before \ heat \ treatment} \tag{9}$$

2.8. Scanning electron microscopy

Scanning electron microscope (SEM)- JSM 6100, JEOL manufactured in Japan, was employed to capture micrographs of raw and fermented LBFs. Moisture free flour particles were placed on carbon sample holders to obtain images at different resolutions at 15 kV of accelerating voltage (Adebiyi et al, 2019). Since observations were taken under high vacuum conditions, metallization of samples was not required.

2.9. Fourier transform infrared (FTIR) spectroscopy

FTIR spectroscopy was undertaken to study the influence of fermentation treatment on structural and molecular interactions within locust bean flours. Tensor 27 spectrophotometer manufactured by Bruker, Germany was employed to obtain the ATR-FTIR spectrum. Scan

was run at 28 °C by placing the flour samples on Attenuated Total Reflectance and peaks were recorded in the range of 4000–600 cm^{-1}. It took 1–2 min to complete the scan (Sadh, Chawla, Bhandari, & Duhan, 2018).

2.10. X-ray diffraction (XRD)

XRD patterns of raw and fermented LBFs were investigated to determine the impact of processing on crystallinity of flour using X-ray diffractometer manufactured by X'pert pro, PAN analytical, Netherland. Diffraction angle (2θ) of 0–50° was undertaken and scanning speed was fixed at 0.05°/minute. Method adopted by González, Vernon-Carter, Alvarez-Ramirez, and Carrera-Tarela (2021) was followed to conduct the analysis.

2.11. Statistical analysis

Experiments were conducted in triplicates i.e. three independent treatments. Calculations were done on dry basis and results were expressed as mean ± standard deviation of replicates. One-way Analysis of Variance (ANOVA) was employed to determine the statistical difference at $p < 0.05$ followed by *post-hoc* tukey's test to confirm the variation between different treatments. Pearson's correlation matrix and Principal component analysis (PCA) and at $p < 0.05$ was developed using XLSTAT. 2021.1 to understand the association between different compositional and functional attributes.

3. Results and discussion

3.1. Anti-nutritional factors

Anti-nutritional factors are non-nutritive deleterious biochemical compounds as they may hinder the absorption and bioavailability of several biomolecules depending upon their concentration within the seeds. Anti-nutrient composition of raw and fermented LBF was assessed in terms of tannin, phytate and saponin content as shown in Table 1. Tannin content of raw LBF was found to be 2.86 mg/g which substantially reduced to 2.22 mg/g after boiling ascribing to combined effect of leaching and heat treatment. Thermal processing of grains could have caused higher leaching loss of tannins in boiling water due to breakdown of protein-tannin complex as reported by (Villacrés et al., 2020). Tannin content further lowered linearly with the treatment time to as low as 1.03 mg/g after 72 h of treatment which could be ascribed to bacterial synthesis of tannase during fermentation (Khan, Karnpanit, Nasar-Abbas, Huma, & Jayasena, 2018). Phytate content of raw LBF was noted as 12.59 mg/g which followed similar trend as that of tannins, linear decline with increasing period of fermentation. Most pronounced effect was observed after 72 h of treatment causing ~60% reduction with reference to raw flour. Degradation of phytate could be ascribed to two major factors including hydrothermal treatment given prior to

fermentation as supported by Castro-Alba et al. (2019) and production of phytase by microbial strains in addition to activation of endogenous phytase during fermentation process due to favourable conditions. Further, Baye, Mouquet-Rivier, Icard-Vernière, Rochette, and Guyot (2013) argued the dependence of phytase action on pH, which explained the higher reduction rate after 48 h of treatment when pH was comparatively low. Saponin content of raw LBF was found as 6.87 mg/g which reduced significantly ($p < 0.05$) by ~28% after hydrothermal treatment, onset of fermentation further reduced it by ~54–72% from 24 to 72 h of treatment. This could be attributed to glycosylation process of fermenting microorganisms and enhanced β-glucosidase activity which reduces the chemical stability of aglycones by splitting the sugar side chains of triterpenoid and steroid saponins, thus lowering their water solubility, consequently saponins lose their toxicity (Bolívar-Monsalve, Ceballos-González, Ramírez-Toro, & Bolívar, 2018; Lai, Hsieh, Huang, & Chou, 2013).

3.2. Bioactive constituents and antioxidant potential

Plant based phytochemicals are often broadly diversified into phenolic acids, polyphenols and flavonoids depending upon their structural and compositional similarities. Total phenolic content of raw LBF was found as 223.65 mg GAE per 100 g which reduced significantly ($p < 0.05$) to 68.99 mg GAE/100 g after boiling treatment (Table 1) which could be attributed to leaching loss in steep water and thermo-degradation of heat sensitive compounds. However, notable increase was later observed during fermentation period from 24 to 72 h, approximately 3-fold increment in comparison to unprocessed flour. This could be ascribed to higher enzymatic activity by xylanase, amylases, proteases and glycosidase which are involved in the degradation of protein-polyphenol complex liberating the insoluble bound polyphenolic compounds (Saharan, Sadh, Duhan & Duhan, 2017). Likewise, 1.5–6.3-fold increment was also noted in total flavonoid content of LBF during fermentation period of 24–72 h; however, insignificant ($p < 0.05$) decline from 53.32 to 49.81 mg QE/100 g flour was noted after boiling. Similar effects on phytochemical constituents were also observed by (Saharan, Sadh, Duhan, & Duhan, 2020) after solid state fermentation of legumes. Since most of the bioactive constituents are compartmentalized in the outer layers of the seeds, many of them are present as complexed to cell wall constituents and certain protein bodies. Keeping in view the fact that only seed surface was exposed to fermentation, it may further be ascertained that enhanced bioactive profile was due to a) enzymatic breakdown of cell wall material which released the polyphenolic compounds freely available for extraction, b) protein hydrolysis due to enzymes which degraded the protein-polyphenolic complexes and their biosynthesis. The results are consistent with findings of Adetuyi and Ibrahim (2014).

Antioxidant activity from natural dietary sources has been reported to augment the human resistance to several modern-lifestyle diseases arising out of oxidative stress (DJordjevic et al, 2010). DPPH- RSA

Table 1
Effect of fermentation time on anti-nutritional factors, bioactive constituents and associated anti-oxidant activity of Locust bean.

Treatments	Anti-nutritional factors			Bioactive constituents		Anti-oxidant activity		
	Tannins	Phytate	Saponins	TPC	TFC	DPPH· RSA	ABTS·+ RSA	Metal chelation
	(mg TA/g)	(mg PA/g)	(mg/g)	(mg GAE/100 g)	(mg QE/100 g)	(%)	(μmol TE/g)	(%)
R(LB)	2.86 ± 0.16[a]	12.59 ± 0.17[a]	6.87 ± 0.12[a]	223.65 ± 3.29[c]	53.32 ± 1.27[d]	17.28 ± 0.28[d]	97.50 ± 1.38[c]	24.28 ± 0.28[c]
B(LB)	2.22 ± 0.09[b]	11.60 ± 0.28[b]	4.95 ± 0.09[b]	68.99 ± 1.28[e]	49.81 ± 0.98[d]	14.82 ± 0.33[e]	21.57 ± 0.21[e]	15.39 ± 0.42[d]
F(LB) 24h	2.01 ± 0.11[c]	9.45 ± 0.41[c]	3.16 ± 0.15[c]	121.25 ± 2.21[d]	82.69 ± 1.44[c]	21.28 ± 0.63[c]	47.45 ± 1.01[d]	24.48 ± 0.82[c]
F(LB) 48h	1.55 ± 0.19[d]	7.53 ± 0.45[d]	2.19 ± 0.13[d]	495.47 ± 8.67[b]	157.09 ± 1.36[b]	32.44 ± 0.87[b]	99.56 ± 1.78[b]	31.38 ± 1.01[b]
F(LB) 72h	1.03 ± 0.16[e]	5.16 ± 0.59[e]	1.92 ± 0.21[e]	647.04 ± 9.56[a]	333.97 ± 1.92[a]	40.26 ± 0.58[a]	146.88 ± 1.28[a]	39.49 ± 1.22[a]

Results are expressed as mean ± standard deviation (n = 3); Values with different alphabets (a-e) in a column are significantly different at $p < 0.05$.
R(LB)- Raw locust bean, B(LB)-boiled locust bean and F(LB) 24, 48 and 72 h - fermented locust beans for 24 h, 48 h and 72 h, TPC- total phenolic content, TFC- total flavonoid content, DPPH· RSA - 2,2-diphenyl-1-picryl-hydrazyl-hydrate radical scavenging activity, ABTS·+ RSA - 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging activity, TA-tannic acid, PA-phytic acid, GAE-gallic acid equivalent, QE-quercetin equivalent.

measures the ability of ingredient to break the free radical chain due to donation of hydrogen atoms by antioxidant compounds. Raw LBF exhibited 17.28% RSA which increased with fermentation time mainly due to higher content of bioactive constituents. DPPH RSA was found to have significant positive correlation with total phenolic content (r = +0.95, $p < 0.05$) and total flavonoid content (r = +0.96, $p < 0.05$). Similarly, ABTS·+ RSA declined with boiling treatment from 97.50 to 21.57 µmol TE/g; however, highest was noted after 72 h of fermentation (146.88 µmol TE/g). Metal chelation in terms of Fe^{+2} was also assessed which varied between 15.39 and 39.49%; lowest for boiled unfermented flour while highest after 72 h of treatment. Attributing to the chemical structure, flavonoids have been reported as the most potent antioxidants which could easily form metal complexes to hinder metal-induced oxidative reactions and effectively quench peroxyl and hydroxyl radicals Lee, Koo, and Min (2004). It could be inferred that fermentation could impart high antioxidant activity predominantly due to three major reasons i) hydrolytic activity of microbial enzymes degrading the plant cell wall material to release bound polyphenols, ii) transformation and depolymerization of larger polyphenolic compounds due to microbial activity and iii) bioconversion of glucosides to form aglycones which possess comparatively higher antioxidant potential (Hur, Lee, Kim, Choi, & Kim, 2014). Singh et al. (2015) have critically examined and reported similar increase in the bioactive constituents and antioxidant activity of the different cereals and legumes during fermentation.

3.3. In vitro starch and protein digestibility

Hydrolysis of starch is an important property of food ingredients and end products as it dictates their biological functionality (Sharma, Sharma, et al., 2021). IVSD of raw LBF was 5.29 g maltose per 100 g which increased significantly ($p < 0.05$) to 10.02 g/100 g after boiling treatment which could be due to gelatinization of starch granules. Further, linear increase was also observed with reference to fermentation time, reaching to 24.82 g/100 g (~5-fold increment) with 72 h of treatment (Table 2). Ogodo, Ugbogu, Onyeagba, and Okereke (2018) also found linear increment in IVSD of soybean flour when exposed to 0–48 h of natural fermentation. This could be possibly due to fermentation induced changes to the endospermic region hydrolysing the protein molecules resulting in higher access of starch to amylolytic enzymes. Degradation of protein and fibre networking with starch and reduction in antinutritional factors could also have contributed to enhanced IVSD of locust bean (Dhital, Bhattarai, Gorham, & Gidley, 2016).

Dietary performance of protein is referred in terms of amino acid composition and its bioavailability which is usually estimated with IVPD assay. Locust bean is a potential source of quality protein and it was observed that raw LBF exhibited 66.47 g/100 g IVPD which improved with heat treatment and fermentation process. After completion of 72 h of fermentation, IVPD of 74.29 g/100 g was noted (Table 2). This could be ascribed to proteolytic activity of microbial enzymes produced during

Table 2
Effect of fermentation time on *in vitro* starch and protein digestibility of Locust bean.

Treatments	IVSD	IVPD
	(g/100 g)	(g/100 g)
R(LB)	5.29 ± 0.48[e]	66.47 ± 0.21[e]
B(LB)	10.02 ± 1.01[d]	69.58 ± 0.13[d]
F(LB) 24h	14.54 ± 1.21[c]	71.28 ± 0.43[c]
F(LB) 48h	20.26 ± 1.45[b]	72.97 ± 0.32[b]
F(LB) 72h	24.82 ± 1.56[a]	74.29 ± 0.67[a]

Results are expressed as mean ± standard deviation (n = 3); Values with different alphabets (a-e) in a column are significantly different at $p < 0.05$.
R(LB)- Raw locust bean, B(LB)-boiled locust bean and F(LB) 24, 48 and 72 h-fermented locust bean for 24 h, 48 h and 72 h, IVSD-*in vitro* starch digestibility, IVPD-*in vitro* protein digestibility.

Table 3
Effect of fermentation time on color profile of Locust bean.

Treatments	L*	a*	b*	Hue	Chroma	ΔE
R(LB)	79.71 ± 0.12[a]	2.19 ± 0.07[e]	23.75 ± 0.32[a]	84.73 ± 0.34[q]	23.85 ± 0.34[a]	0.00 ± 0.00[e]
B(LB)	71.46 ± 0.08[b]	4.59 ± 0.02[d]	16.64 ± 0.23[b]	74.58 ± 0.23[b]	17.26 ± 0.28[d]	11.15 ± 0.20[d]
F(LB) 24h	67.49 ± 0.11[c]	5.03 ± 0.03[c]	16.98 ± 0.39[bc]	73.50 ± 0.12[bc]	17.71 ± 0.13[d]	14.26 ± 0.34[c]
F(LB) 48h	62.29 ± 0.10[d]	5.81 ± 0.12[b]	17.28 ± 0.13[c]	71.42 ± 0.14[c]	18.23 ± 0.21[c]	18.93 ± 0.18[b]
F(LB) 72h	52.47 ± 0.12[e]	7.98 ± 0.21[a]	18.77 ± 0.23[d]	66.97 ± 0.21[d]	20.40 ± 0.30[b]	28.30 ± 0.45[a]

Results are expressed as mean ± standard deviation (n = 3); Values with different alphabets (a-e) in a column are significantly different at p < 0.05. R(LB)- Raw locust bean, B(LB)-boiled locust bean and F(LB) 24, 48 and 72 h-fermented locust bean for 24 h, 48 h and 72 h, ΔE – overall difference in color profile with respect to raw locust bean.

fermentation. Further, deterioration of protein-polyphenol complex could also have increased the accessibility of proteins to digestive enzymes. Duodu, Taylor, Belton, and Hamaker (2003) also suggested that anti-nutritional factors such as tannins, phytates and saponins biochemically interact and form complex compounds with proteins, thus restricting their digestibility. This relationship could also be signified with the negative correlation between IVPD and tannin content (r = −0.98, $p < 0.05$), phytate content (r = +0.95, $p < 0.05$) and saponins (r = +0.98, $p < 0.05$). Similar increase in the protein digestibility has been reported for soybean highlighting the potential of fermentation enzymes to modulate the functionality of proteins (Ketnawa & Ogawa, 2019).

3.4. Color profile

Fermentation treatment was found to have significant variation ($p < 0.05$) in the tristimulus color parameters i.e. L*, a* and b* values as shown in Table 3. L* value, which indicate the brightness or whiteness of the product, was noticed to decline linearly with fermentation treatment. Raw LBF had L* value of 79.71 while it varied from 71.46 to 52.47

Table 4
Effect of fermentation time on techno-functional properties of Locust beans.

Treatments	WAC	WSI	OAC	SP	GC	EA	ES
	(g/g)	(%)	(g/g)	(g/g)	(mm)	(%)	(%)
R(LB)	3.11 ± 0.08[a]	3.16 ± 0.13[e]	0.85 ± 0.03[e]	4.45 ± 0.13[d]	36.4 ± 0.2[e]	45.07 ± 1.02[a]	26.34 ± 0.34[a]
B(LB)	2.62 ± 0.07[b]	4.54 ± 0.15[d]	0.87 ± 0.02[d]	4.87 ± 0.14[a]	39.7 ± 0.4[d]	14.08 ± 0.88[d]	12.45 ± 0.65[d]
F(LB) 24h	2.53 ± 0.05[c]	6.15 ± 0.31[c]	0.94 ± 0.01[c]	4.71 ± 0.15[b]	46.3 ± 0.5[c]	15.33 ± 0.32[cd]	13.56 ± 0.45[cd]
F(LB) 48h	2.39 ± 0.10[d]	7.94 ± 0.32[b]	0.97 ± 0.03[b]	4.58 ± 0.09[c]	50.2 ± 0.5[b]	16.54 ± 0.43[bc]	14.67 ± 0.25[bc]
F(LB) 72h	2.10 ± 0.11[e]	10.41 ± 0.21[a]	1.01 ± 0.02[a]	4.20 ± 0.19[e]	55.4 ± 0.8[a]	17.15 ± 0.34[b]	16.55 ± 0.42[b]

Results are expressed as mean ± standard deviation (n = 3); Values with different alphabets (a-e) in a column are significantly different at p < 0.05. R(LB)- Raw locust bean, B(LB)-boiled locust bean and F(LB) 24, 48 and 72 h-fermented locust bean for 24 h, 48 h and 72 h, WAC- water absorption capacity, WSI- water solubility index, OAC- oil absorption capacity, SP- swelling power, GC- gel consistency, EA-emulsion activity and ES-emulsion stability.

during fermentation period of 0–72 h. Similar changes were also seen in chromaticity coordinates i.e. a* and b* values after fermentation which varied from 2.19 to 7.98 and 23.75 to 18.77 respectively. The results are consistent to the finding of Olukomaiya et al. (2020). It may be inferred that alkaline fermentation darkened the resultant flour mainly due to oxidative and enzymatic reactions. Increasing a* values indicated greenish tinge in the fermented flour while declining b* values dictated dominance of yellowish character over blue. Overall color difference (ΔE) suggested that prolonged fermentation time significantly varied the tristimulus properties, most pronounced effect was seen after 72 h of fermentation. The changes in the color profile could be due to oxidation reactions occurring with the accelerated microbial action in addition to Strecker degradation of amino acids and Maillard series of reactions during post fermentation treatment such as drying. The findings are consistent with the results of Diez-Simon, Eichelsheim, Mumm, and Hall (2020). Darkening of the brown color of fermented locust bean (*dad-dawa*) could be ascribed to breakdown of amino acids, which is also responsible for the ammoniacal odor, and other polyphenolic constituents.

3.5. Techno-functional properties

Functional characteristics are physico-chemical properties of food ingredients reflect their suitability for use in finished products. Effect of fermentation time on various functional properties of LBF is presented in Table 4. Raw LBF exhibited 3.11 g/g of WAC which reduced significantly during fermentation treatment. After 72 h of fermentation, WAC reduced to 2.10 g/g which could be due to structural breakdown of protein and starch molecules by digestive enzymes. The results are consistent with the findings of the Olukomaiya et al. (2020). This could be attributed to lower availability of hydrophilic groups in fermented LBF, signifying its potential to use in food formulations in thin gruels. Further, WSI was found to increase significantly ($p < 0.05$) from 3.16 to 10.41% with progression of treatment time. Degradation of large molecular weight compounds into smaller fractions during fermentation

could resulted in higher soluble fragments. The results are in agreement with Sharma and Sharma (2022) who reported similar changes in the functional properties of foxtail millet flour during fermentation. Structural variation in proteins also exposed hydrophobic regions of LBF, as a result, significant ($p < 0.05$) increment in OAC was noted from 0.85 to 1.01 g/g. Interestingly, SP of LBF increased with boiling (4.45–4.87 g/g); however, later declining trend was observed with fermentation time from 0 to 72 h (4.87–4.20 g/g). Yuan, Lu, Cheng, and Li (2008) also noted lower SP of fermented corn starch and stated that it is property of mainly amylopectin units while amylose may dilute or inhibit swelling, specially amylose-lipid complex hinders the swelling potential of flours. Breakdown of starch into shorter filaments further reduced the gelling ability as depicted by increment in GC from 36.4 mm to 55.4 mm due to fermentation which could be due to reduced viscosity of gels after fermentation as reported by Adiandri and Hidayah (2019). Emulsification potential of flours is a function of solubility, structure and concentration of proteins in addition to their flexibility and exposure of hydrophobic moieties (Abd Elmoneim et al, 2005). Severe heat treatment during boiling disrupted the protein structure, as a result EA and ES reduced significantly; however, fermentation improved the emulsifying properties with extended treatment time, thus it could be hypothesized that fermentation imparted hydrophobic property to LBF due to activity of proteolytic enzymes. The current findings are also supported by Kumitch, Stone, Nickerson, Korber, and Tanaka (2020). Further, Chawla, Bhandari, Sadh, and Kaushik (2017) also found similar improvement in the emulsification potential of the fermented flour due to changes in the hydrophilic-lipophilic balance as a result of enzymatic hydrolysis.

3.6. Macromolecular morphology using scanning electron microscopy

Scanning electron micrographs revealed the macromolecular arrangement of LBF as influenced by boiling and fermentation treatments. Raw flour exhibited intact starch granules and distinguished protein matrix as identified by encircled regions and pointing tips of
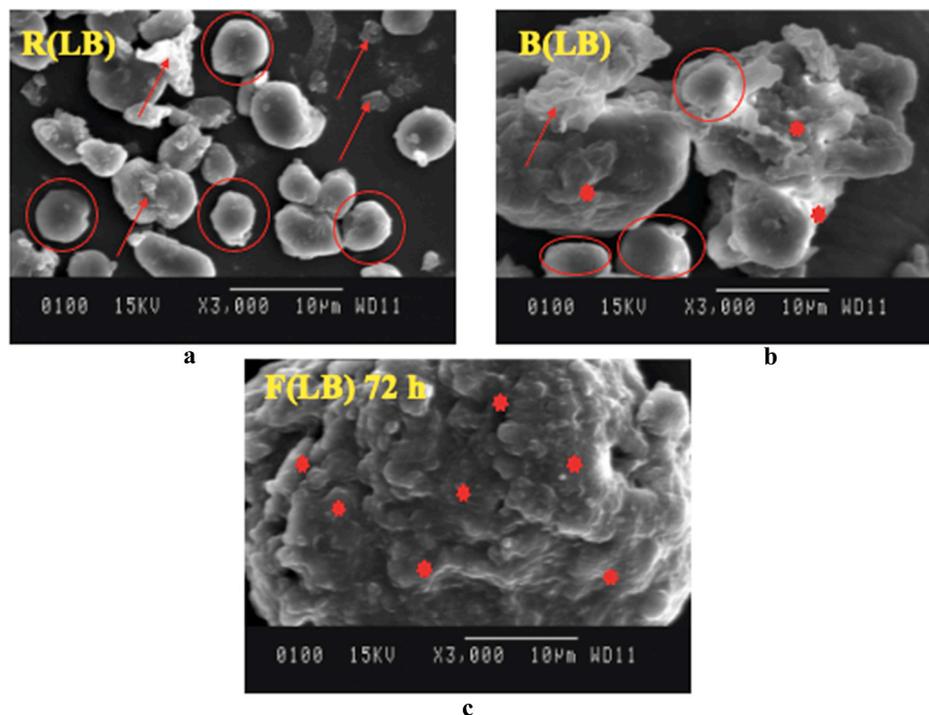
Fig. 1. Scanning electron micrographs of R (LB)- Raw locust bean flour, B (LB)-boiled locust bean flour and F (LB) 72 h-fermented locust bean flour −72 h at 3000 × ; encircled regions signify native starch granules while arrows point towards protein matrix and stars indicate gelled three dimensional networking with no distinguished boundary between starch and protein matrix.

(a)

(b)

(c)
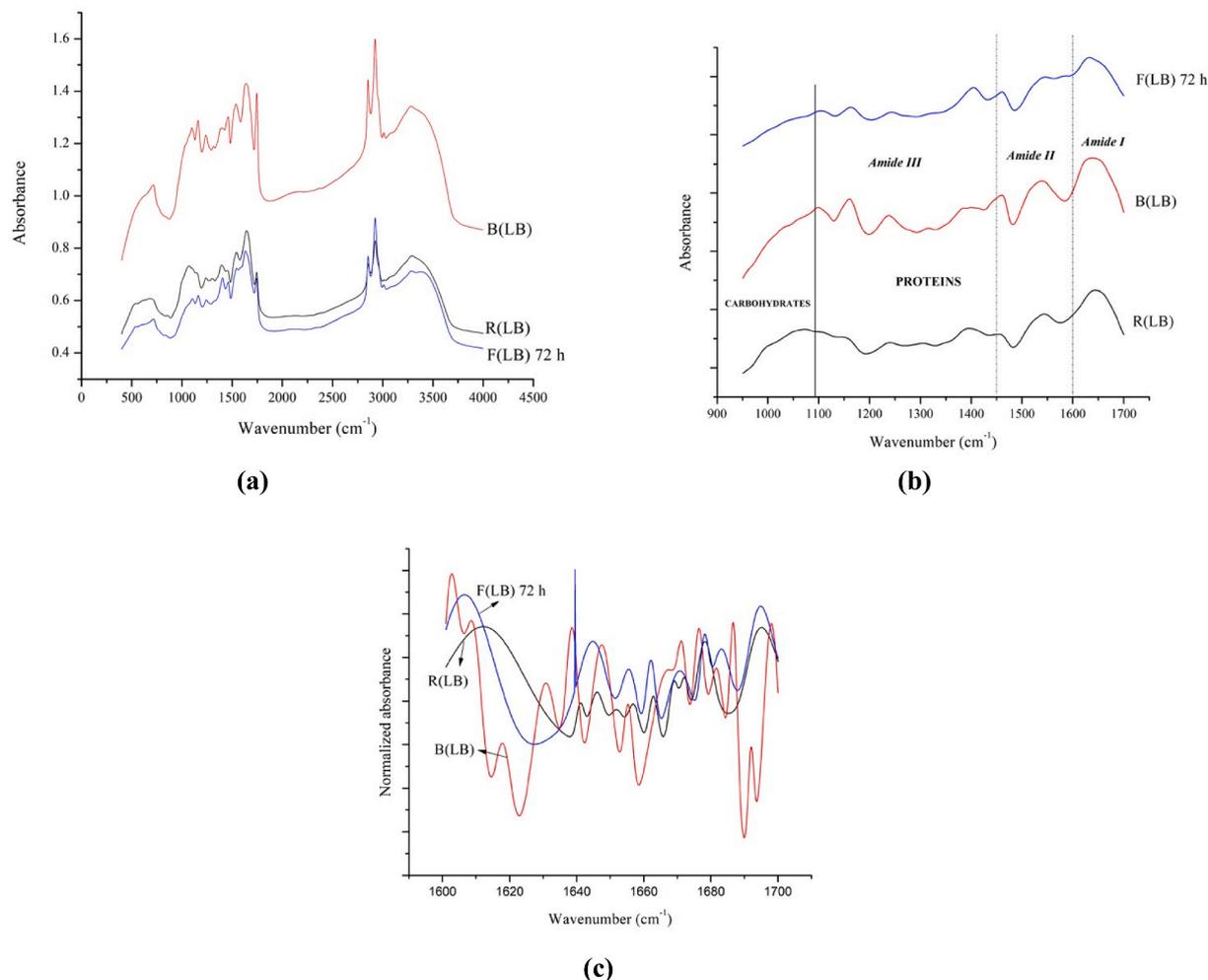
Fig. 2. (a) ATR-FTIR spectra between 400 and 4000 cm^{-1} (b) FTIR spectrum signifying changes in the carbohydrate and protein regions and (c) Gaussian deconvolution of second derivative of absorbance in amide II regions to analyze secondary protein structure; R (LB)- Raw locust bean flour, B (LB)-boiled locust bean flour and F (LB) 72 h-fermented locust bean flour −72 h..

arrows respectively in Fig. 1a while boiling resulted in gelatinization of starch granules (Fig. 1b). Gelatinized LBF could be characterized by disrupted starch granules, more likely as a compact mass from three-dimensional gel networking as shown in Fig. 1b. Huang et al. (2017) also stated that gelatinized starch fractions deform after swelling which later merge and develop a network by fusion at several junctions. Since gelatinized starch is prone to enzymatic degradation, microbial enzymes brought significant variation in morphology of flour particles as seen in Fig. 1c where granular integrity of starch molecules could not be identified after 72 h fermentation treatment. Handoyo and Morita (2006) also reported disintegration of starch granules due to action of different hydrolytic enzymes such as amylases, proteases and lipases produced by fermenting microorganisms. It was further observed that in addition to targeted substrates, cell wall constituents were also hydrolyzed to produce thick uniform mass with no visible boundary between flour constituents.

3.7. Macromolecular characteristics and secondary protein structure

FTIR spectrum featured the raw, boiled and fermented LBFs and revealed significant variation in the peak intensities at various bands; however, no newer groups or derivates were produced during 72 h of fermentation as shown in Fig. 2a. FTIR region of 900–1100 cm^{-1} belongs to carbohydrates and 1100-1700 cm^{-1} characterizes proteins with amide I, amide II and amide III regions as shown in Fig. 2b. FTIR peak at

1047 cm^{-1} is related to ordered structure or crystalline nature while 1022 cm^{-1} corresponds to amorphous fraction (Yang & Tao, 2008), thereby absorbance ratio at 1047:1022 cm^{-1} was found as 1.04 for raw, 1.02 for boiled and 1.01 for fermented flour which means crystallinity of LBF was slightly affected by bioprocessing treatment. Further, secondary structure of proteins was studied after Gaussian deconvolution of second derivative data of absorbance in amide II region between 1600 and 1700 cm^{-1} as shown in Fig. 2c. Peaks in the 1600–1640 and 1670-1680 cm^{-1} corresponded to β-sheet structure, 1640-1650 cm^{-1} to random coils, 1650-1660 cm^{-1} to α-helix and 1660–1670,1680-1700 cm^{-1} to β-turn structure. It was observed that β-sheet structure prevailed in raw and treated LBFs while its proportion reduced from ~55% to ~48% after 72 h of fermentation. Further, random coil structure increased form 5%–12% for boiled and 15% for fermented sample. Similarly, fermentation reduced the proportion of β-turn structure from 34 to 29% while α-helix structure increased from 4 to 6%. The peak at 857 cm^{-1} present only in case of raw LBF could be attributed to undamaged starch granules. The results are also similar to findings of Correia, Nunes, Duarte, Barros, and Delgadillo (2005).

3.8. X-ray diffraction

XRD patterns of raw, boiled and fermented LBF are shown in Fig. 3. Hydrothermal treatment prior to fermentation caused notable variation in the crystallinity of the LBF while minimal impact was imparted by
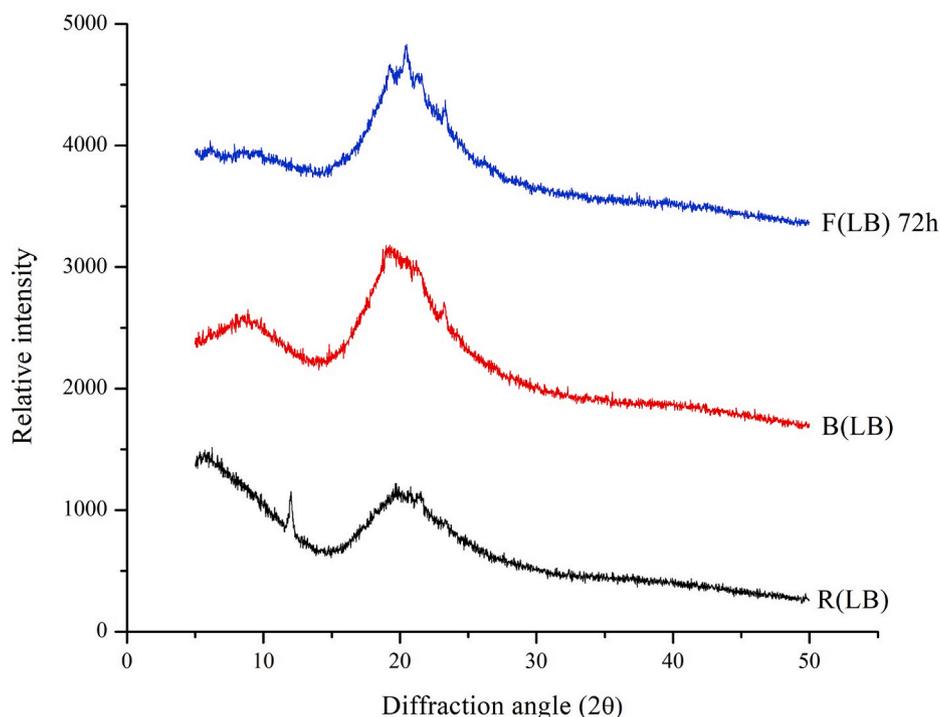
Fig. 3. X-ray diffraction patterns of R (LB)- Raw locust bean flour, B (LB)-boiled locust bean flour and F (LB) 72 h-fermented locust bean flour −72 h..
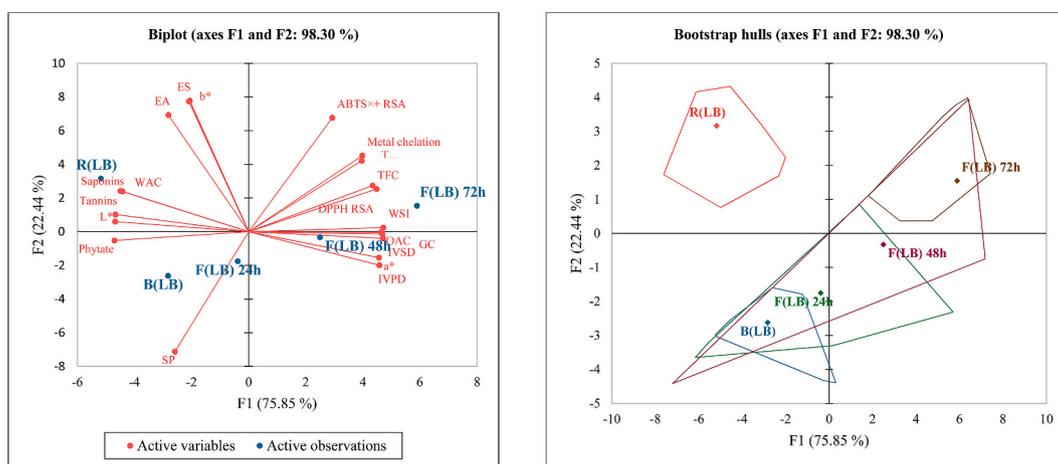
Fig. 4. Multivariate analysis (PCA) defined by two factors (F1-75.85% and F2-22.44%) with data variance of 98.30% Active variables represent different compositional and functional parameters and active observations indicate different treatments.

fermentation. Sharp peak was noted in raw LBF at 2θ = around 12.5 which could be due to amylose-lipid complex (Guo, Yu, Copeland, Wang, & Wang, 2018); however, heat treatment must had degraded that complex as no such peak was detectable in boiled and fermented LBF. High intensity peak in fermented LBF at around 20 is a typical characteristic of type V starch which is generally observed in modified starch samples featuring the loss of crystallinity (Purohit, Jayachandran, Raj, Nayak, & Rao, 2019). Interference of surface lipids and protein among starch molecules also influence the impact of fermentation on crystallinity of starch granules (Park, Sung, Choi, & Park, 2020), thus minimal impact of fermentation could been in X-ray diffractograms.

3.9. Principal component analysis (PCA)

Data were subjected to multivariate analysis using PCA to validate the variation among different time periods of fermentation from 0 to 72

h with reference to raw LBF. Data variance of 98.30 was explained by two factors F1 and F2 as 75.85% and 22.44% respectively as shown in Fig. 4. Squared cosines of LBF fermented for 24 h was detailed by F2 while others corresponded with F1. Further, squared cosines of variables revealed that F2 was well correlated with ABTS + RSA, ES, EA, SP and b* values while other parameters were explained by F1. Specific inferences of the PCA are as:

- First quadrant was equipped with raw LBF with variables including tannins, saponins, WAC, L*, b* EA and ES.
- Boiled and 24 h fermented LBFs were present in second quadrant with phytate content and SP.
- Third quadrant containing 48 h fermented LBF exhibited OAC, GC, IVSD, IVPD, and a* in close proximity to each other signifying higher Pearson's correlation coefficient.

- WSI, antioxidant assays (DPPH RSA, ABTS + RSA, metal chelation) and bioactive constituents (Total phenolic and flavonoid content) presented higher correlation in fourth quadrant which were best associated with 72 h fermented LBF.

4. Conclusion

Present investigation confirmed that antinutritional factors in locust bean reduced significantly in linear relationship with fermentation time which further enhanced the *in vitro* digestibility of proteins and starch. It could be ascribed to the fact that protein-polyphenol complex formation was prevented resulting in the better access of macromolecular to hydrolytic enzymes during digestion. Since bioactive compounds are compartmentalized in the outer layers of seeds, cell wall degradation during fermentation assisted in release of bound polyphenolic compounds along with their bio-synthesis which favoured the antioxidant potential of locust bean flours. Further, breakdown of starch and proteins during fermentation altered the technological properties; fermented locust bean flours could hold less water and swelling power also reduced substantially. Fermentative breakdown of macromolecules drastically lowered the gelling potential of locust bean flour which could be dedicated to disruption of granular integrity of starch molecules and their native morphological characteristics. Changes in the secondary protein structure as found in FTIR analysis altered the hydrophobic-hydrophilic interactions and consequently, fermented flours exhibited higher oil absorption capacity and varied surface properties. It could be ascertained that 72 h of fermentation treatment brough most significant changes in the techno-bio-functionality of locust bean flour.

CRediT authorship contribution statement

Caleb Maina Yakubu: Data curation, Formal analysis, Methodology, Writing – original draft. **Rajan Sharma:** Conceptualization, Formal analysis, Writing – original draft, Writing – review & editing. **Savita Sharma:** Conceptualization, Project administration, Supervision, Writing – review & editing. **Baljit Singh:** Conceptualization, Supervision, Writing – review & editing.

Declaration of competing interest

None.

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