การหมักเมล็ดงาม้อน เพิ่มการนำไปใช้ทางชีวภาพ และชีวภาพพร้อมใช้ของแร่ธาตุให้ดีขึ้น โดยการลดระดับสารต้านสารอาหาร

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บทคัดย่อ

การขาดแร่ธาตุเป็นสาเหตุสำคัญที่นำไปสู่ปัญหาสุขภาพของประชากรทั่วโลก อย่างไรก็ตาม สามารถแก้ไขได้โดยการปรับเปลี่ยนองค์ประกอบทางเคมีของอาหาร หรือด้วยวิธีการทางเทคโนโลยีการอาหาร รวมทั้ง การใช้กระบวนการหมักด้วยจุลินทรีย์ เพื่อลด/กำจัดสารที่ขัดขวางการนำไปใช้ได้ของสารอาหาร และเพิ่มการนำไปใช้ได้ของแร่ธาตุในอาหาร โดย เมล็ดงาม้อนอุดมไปด้วยแร่ธาตุหลายชนิด แต่ประกอบด้วยสารต้านสารอาหาร เช่น ไฟเตต และออกซาเลต เช่นกัน การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาผลของการหมักต่อปริมาณสารต้านสารอาหาร และการนำไปใช้ทางชีวภาพ รวมทั้งชีวภาพพร้อมใช้ของแร่ธาตุในเมล็ดงาม้อน เมล็ดงาม้อนหมักถูกวิเคราะห์การนำไปใช้ได้ทางชีวภาพ และชีวภาพพร้อมใช้ของแร่ธาตุ ด้วยวิธี ไดแอลลิซิส (dialysis) และ Caco-2 cells ตามลำดับ สารต้านสารอาหารถูกวิเคราะห์โดยใช้ Assay kits เปรียบเทียบกับเมล็ดงาม้อนที่ไม่ผ่านการหมัก ผลการศึกษา พบว่า การหมักมีผลทำให้ปริมาณไฟเตต และออกซาเลตในเมล็ดงาม้อนหมัก ลดลงจาก 1.56 ± 0.14 กรัม/100 กรัม และ 0.98 ± 0.09 มิลลิกรัม/100 กรัม เหลือ 0.50 ± 0.02 กรัม/100 กรัม และ 0.58 ± 0.08 มก./100 กรัมตามลำดับ ส่งผลทำให้การนำไปใช้ทางชีวภาพของแคลเซียมและเหล็กในเมล็ดงาม้อน มีค่าเพิ่มขึ้นอย่างมีนัยสำคัญจาก 16.29 ± 0.93% และ 11.87 ± 0.37% เป็น 19.77 ± 0.33% และ 16.32 ± 0.39% ตามลำดับ นอกจากนี้ ยังพบว่า ชีวภาพพร้อมใช้ของแคลเซียม และเหล็กในเมล็ดงาม้อนหมักมีค่าเพิ่มขึ้น 1.25 และ 1.4 เท่า ตามลำดับอีกด้วย เมล็ดงาม้อนหมักควรจะถูกนำไปใช้ในการพัฒนาผลิตภัณฑ์อาหาร ในกลุ่มผลิตภัณฑ์เสริมอาหาร อาหารเพื่อสุขภาพ และโภชนเภสัช

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Perilla Seed Fermentation Improves Minerals Bioaccessibility and Bioavailability with Reduced Antinutrients Content

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Abstract

Mineral deficiency is a significant cause of health problems worldwide. However, it can be overcome by modifying the food's chemical composition or food technological means including utilization of microbial fermentation process to enhance the nutrients' bioavailability and removal/reduction of undesirable components. Perilla seeds are rich in dietary minerals, but they also contain antinutrients such as phytate and oxalate. This study aimed to investigate the effects of perilla seeds fermentation on antinutrients content and minerals bioaccessibility and bioavailability. The fermented perilla seeds (FPS) was determined for bioaccessibility and bioavailability of mineral by using the dialysis method and Caco-2 cell models, respectively. The antinutrients in FPS were analyzed by assay kits and compared with unfermented (untreated) perilla seeds. The result show that fermentation significantly reduced the levels of phytate and oxalate in FPS from 1.56 ± 0.14 g/100 grams and 0.98 ± 0.09 mg/100 grams to 0.50 ± 0.02 g/100 g and 0.58 ± 0.08 mg/100 g, respectively. This resulted in a significant increase in the bioaccessibility of Ca and Fe in FPS from 16.29 ± 0.93% and 11.87 ± 0.37% to 19.77 ± 0.33% and 16.32 ± 0.39%, respectively. Furthermore, the bioavailability of Ca and Fe from FPS was found increased by 1.25- and 1.4-folds, respectively. FPS should be utilized in the development of dietary supplement, functional foods and nutraceuticals with improved dietary value.

Keywords: Antinutrients, Bioavailability, Perilla seed fermentation, Minerals, Bioaccessibility, Food Security

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Introduction

The prevalence of mineral deficiency among several countries of the world including Thailand is associated with various serious health issues today[1]. Among numerous crucial biological functions of minerals in human body, few of them include the synthesis and activation of enzymes and hormones, sustenance of normal blood levels, regulation of muscle and nerve functions, production of neurotransmitter, regulation of key pH levels in body fluids for metabolic response control, maintenance of osmotic equilibrium between the cell and its surroundings[2]. Lack of essential minerals might cause serious metabolic problems and jeopardize the organism's health[3]. Apart from mineral availability, mineral absorption by intestinal cells is another crucial factor that influences the processing and maintenance of adequate mineral balance in the body[4]. In this regard, economical, safe, healthy and sustainable technological methods for food processing to alter the chemical composition of food matrix as well as the elimination of undesired components such as antinutrients, are employed. It has been shown in previous studies that among various food processing techniques, fermentation process significantly controls the nutrient quality and uptake by reducing the antinutrients components in food[5].

Perilla frutescens, commonly called perilla, is an annual herbal plant belonging to the mint family (*Lamiaceae*) and is usually used as a functional food and herbal medicine in Asian countries. The plant is known to belong originally to the East Asian countries[6] such as China, Korea, and Japan. In Thailand, perilla is grown in northern areas, particularly Phayao, Chiang Rai, Chiang Mai, Phrae, Nan, Lampang, Lamphun and Mae Hong Son provinces. Perilla seeds, also known as “Nga-Kee-Mon” in Thailand, are known to possess high culinary and nutritional value and are widely used as functional dietary supplements in food industry. Perilla seed has been used as an indigenous food for a long time, pounded and mixed with sticky rice, called “Kao-Nuk-Nga”. The seeds are good source of essential fatty acids, for example, alpha-linolenic acid; aminolevulinic acid, protein, carbohydrate, vitamins, and minerals especially calcium, phosphorus, and magnesium[7]. Furthermore, several studies have reported the biological properties of perilla seed extracts including antioxidant[8], antidepressant[9], anti-asthmatic[10] and anti-hypersensitivity[11]. Because of these properties, perilla seeds have recently grown popularity for cosmetic, food, and therapeutic uses.

Fermentation is a well-known traditional food processing technique that is used to improve safety, nutritional value, sensory qualities and shelf life of food [12]. Based on previous studies, fermentation has been found to have a positive effect on reducing antinutrients in various food products. In the case of African black nightshade and African spider plant, lactic acid fermentation was shown to significantly reduce anti-nutrients[9]. In 2013, Adeyemo and Onilude demonstrated that the fermentation of soybeans with *Lactobacillus plantarum* substantially reduced the phytate levels, from 1.16 to 0.04 mg/g. Additionally, a recent study by Ningwei et al. in 2022 supported these findings by showcasing the significant decline in both oxalic acid and phytic acid content during the fermentation process in paper mulberry silage [13]. These observations suggest that microorganisms, particularly *L. plantarum*, plays a crucial role in breaking down phytate and oxalate, potentially enhancing the bioavailability of essential dietary nutrients[14].

Perilla seeds are considered rich in calcium (Ca) and iron (Fe), but they also contain compounds like phytate and oxalate that can inhibit the bio absorption of these minerals. However, the effect of fermentation on antinutrients levels in association with mineral bioaccessibility and bioavailability of perilla seeds has not been reported yet. The present study aimed to examine the effect of fermentation on antinutrients of perilla seeds as well as mineral bioaccessibility and bioavailability, to improve the dietary features of perilla seeds that could be particularly useful for future applications.

Materials and Methods

Sample preparation and fermentation of seeds

Perilla seeds were obtained in February 2023 from Project Baan Lek Nai Par Yai, a Royal initiative located in Pa Daet Subdistrict, Mae Suai District, Chiang Rai Province. The seeds were divided into two groups, one group of seeds fermented with *Lactobacillus spp*., that was obtained from *Musa Sapientum* Linn, at room temperature for 72 h was referred as fermented perilla seeds (FPS), while the other untreated group of seeds referred as unfermented perilla seeds (UPS) was used as a control. In the fermentation process, perilla seeds and *Lactobacillus spp.* were correspondingly utilized in a ratio of 8:2. The fermentation was conducted in a laboratory that maintains biosafety standards according to the Good Hygiene Practices (GHP) standard, ensuring the safe handling of all materials and microorganisms.

Anti-nutrients analysis

(1) Phytate analysis

Phytate content (total phosphorus) was measured using a phytic acid assay kit (K-PHYT) (Megazyme, Wicklow, Ireland) as described earlier by McKie and McCleary (2016) [15], with results expressed as g (phytate)/100 g of FPS or UPS. Briefly, the dried samples of FPS and UPS (1 g) were used and extracted in 20 mL of 0.66 M HCl for 3 h., The supernatant was analyzed using K-PHYT according to the manufacturer's instruction to determine the quantity of inorganic phosphate and the total inositol phosphorus content through a colorimetric reaction. The measurement was performed in the UV/vis spectrophotometer *Jasco V-530* at 655 nm within 3 h.

(2) Oxalate analysis

Extraction tests were conducted using a container containing 1.0 g of the sample and 50 mL of deionized water. This setup was submerged in a water bath at 70 °C for a duration of 180 min[16]. Oxalate content was measured using an oxalate assay kit (MAK315, Sigma-Aldrich, USA) as described previously by Anna K. et al., 2020[17], with results expressed as mg (oxalate)/100 g of FPS or UPS.

Determination of Ca and Fe bioaccessibility

Bioaccessibility of both Ca and Fe in perilla seeds was assessed via *in vitro* method, i.e., dialyzability, by simulating the peptic and pancreatic digestion processes according to Ting and Loh, (2016)[18]. Peptic and pancreatic digestion were simulated by the enzyme pepsin (P7000, from porcine stomach mucosa), pancreatin (P1750, from porcine pancreas) and bile extract (B8631, porcine). Mineral contents of the dialysates were analyzed by Atomic Absorption Spectroscopy. Finally, the bioaccessibility of these minerals was calculated using the given formula:

$$\%\text{Bioaccessibility} = Y/Z \times 100\%$$

Where

Y= dialysate portion of Ca or Fe (g mineral/ 100 g of sample)

Z= total Ca or Fe content (g mineral/100 g of sample)

Analysis of Ca and Fe bioavailability

Bioavailability of Ca and Fe was performed using the *in vitro* Caco-2 cell model as described previously by Garrett et al., 1999[19]. Briefly, the following steps (1-4) were followed to complete the procedure:

(1) Simulated gastric digestion

The samples, weighing between 0.7-1.0 g, were placed into a 50 mL screw-

cap polypropylene tube. Solution A, consisting of 120 mM NaCl, 6 mM $CaCl_2$, and 5 mM KCl, was added to achieve a final volume of 30 mL per reaction (30 mL/Rx volume), and the mixture was thoroughly stirred. The pH was then reduced to 2.5 ± 0.1 using 1 M HCl, noting the volume of acid needed. Subsequently, 2 mL of a pepsin stock solution (40 mg/mL in 100 mM HCl) was added to each tube, resulting in a final pepsin concentration of 2 mg/mL. The volume was further increased to 40 mL/Rx using solution A, maintaining the pepsin concentration at 2 mg/mL. The sample was then covered with nitrogen gas and the tube was sealed. Finally, the samples were incubated in a shaking water bath at 37 °C and 60 rpm, for an hour before further analyses.

(2) Simulated small intestinal digestion

After 1 h of incubation, the tubes were removed from the water bath followed by addition of 1M $NaHCO_3$ to elevate the pH to 6.0 ± 0.2, and the volume introduced was recorded. Next, 3 mL of a bile extract stock solution (40 mg bile extract/mL in 100 mM $NaHCO_3$, resulting in a final concentration of 2.4 mg/mL) was added to each tube. Subsequently, 2 mL of pancreatin-lipase stock solution was introduced, which was prepared with 10 mg pancreatin and 5 mg lipase/mL in 100 mM $NaHCO_3$. The pH was then adjusted to 6.5 ± 0.1 using 1M NaOH. The final volume was increased to 50 mL using solution A, achieving final concentrations of bile extract, pancreatin, and lipase at 2.4, 0.4, and 0.2 mg/mL, respectively. The sample was covered with nitrogen gas, and the tubes were sealed. Finally, the sample was incubated in a shaking water bath at 60 rpm for 2 h at 37 °C.

(3) Preparation of micelle or aqueous fraction

The tubes were removed from the water bath. The samples, referred to as the digesta, were thoroughly mixed, covered with nitrogen, and a portion was transferred to a centrifuge tube. Tubes were then blanketed with nitrogen and sealed, followed by centrifugation at 6,500 rpm for 60 min at room temperature. After centrifugation, the aqueous fraction was collected and filtered with 0.2 μm filter membrane. The filtrate was transferred to a clean tube labeled as the micelle fraction. If cell uptake was to be studied, a portion of this fraction was diluted with cell culture medium. The remaining fraction was covered with nitrogen and stored at -20°C for further analysis. Additionally, a portion of the digesta was transferred to another tube, blanketed with nitrogen, and stored at -20°C until further analysis.

(4) Cell Culture

Human colon adenocarcinoma cells (Caco-2 cell) were purchased from American Type Culture Collection (ATTC, Rockville, MD). Cultures of Caco-2 cells were used between passage number 24 and 30. Cells were grown in 75-T flask (75 cm2 cell culture flask) and maintained with complete medium. The complete medium contained with Dulbecco's modified Eagle's medium (DMEM) (D7777; Sigma Chemical Co. USA), 15% (v/v) heat-inactivated FBS for pre-confluence and 7.5% (v/v) heat-inactivated FBS for post-confluence, 1% (v/v) 200 mM L-glutamine, 1% (v/v) nonessential amino acids, 1% (v/v) penicillin-streptomycin (anti-biotic) and 0.2% (v/v) fungizone (antifungal) at 37°C in a humidified incubator (model 3111, USA) atmosphere of 95% air/5% CO_2.

(5) Permeation of micellar calcium or iron by Caco-2 human intestinal cells

Experiments for examining permeation or transportation of Ca and Fe were performed on the same day with triplicate cultures to facilitate direct comparison of results. Caco-2 cells were seeded in trans-well inserts of 6-well plates (ThinCertsTM-TC Einsatze, Greiner Bio-one, Switzerland) at a density of 3.0 x 10^4 cells/2 mL of complete medium/well in an apical compartment. A basolateral compartment was added with 2 mL of phenol red-free DMEM. Cells were incubated at 37 °C in a humidified atmosphere of 95% air/5% CO_2. Fresh complete medium was changed every 2 days. At 21-24 days, by achieving the confluence with the trans epithelial electrical resistance (TEER) > 500 Ω/cm^2, when Caco-2 cells could exhibit enterocyte characteristics during this period as assessed by the activities of the marker enzymes alkaline phosphatase and sucrase including the tight junction for transportation[20], differentiated monolayers were washed with DMEM before adding 2 ml of the test medium containing the micelle fraction that was diluted 1:3 (v/v) with basal DMEM. Monolayers were incubated at 37 °C for 4 h. Solution from apical and basolateral compartment were collected for determination of the amount of Ca and Fe. The % bioavailability of minerals was calculated using the given formula:

$$\%\text{Bioavailability} = X/Y \times 100\%$$

Where

X= Bioavailable fraction (mg mineral/100 g)

Y= Test medium (mg mineral/100 g)

Data analysis

Statistical analysis was calculated using the SPSS program version PASW

statistic 18. All experiments were performed in triplicate (n = 3) and expressed as the mean ± standard deviation. A comparison of the mean of the results of each experiment between FPS and UPS was analyzed by using an unpaired t-test. *P-value* < 0.05 was considered statistically significant.

Results

Assessment of antinutrients' level in FPS

The antinutrient contents in FPS were measured and compared to those in UPS in this study by utilizing assay kits known for their accuracy. In this regard, a significant reduction in phytate content in FPS was noted when compared to that of UPS as shown in Table 1. To quantify the phytate content, a calibration curve was established with the phosphorus calibration curve having the equation y = 0.0827x + 0.0003 and R^2 = 0.9916. The phytate content in UPS was 1.56 ± 0.14g/100 g, while in FPS, the phytate level had a significant decline to 0.5 ± 0.02 g/100 g of FPS weight, ($p < 0.05$ as shown in Table 1). Similarly, the oxalate content was found to be decreased from 0.98 ± 0.09 mg/100 g in UPS to 0.58 ± 0.08 mg/100 g in FPS. These results suggested that the fermentation process has a significant effect on reducing the levels of antinutritional components, specifically phytate and oxalate, in perilla seeds.

Table 1. Anti-nutrient contents of unfermented and fermented perilla seeds.

Formulation	Anti-nutrients	
	Phytate (g/100 g)	Oxalate (mg/100 g)
UPS	1.56 ± 0.14^a	0.98 ± 0.09^a
FPS	0.50 ± 0.02^b	0.58 ± 0.08^b

***** UPS is unfermented perilla seeds and FPS is fermented perilla seeds. All data are shown as the mean ± standard deviation (SD) of triplicate determination (n = 3). Values with the same letter within the same column are not significantly different at $p < 0.05$.

Minerals' bioaccessibility analysis via dialysis method

In this section, our investigative focus remained on the bioaccessibility of Ca and Fe in perilla seeds, via dialysis method to solve the complex dynamics of mineral availability after fermentation process. The dialysis method is a well-

established *in vitro* technique known for imitating human gastrointestinal conditions[21] to provide a foundation for the subsequent practical analysis.

The results from the dialysis method were notable regarding the bioaccessibility of Ca and Fe in FPS, as shown in Table 2 and Figure 1. The Ca content in UPS were around 450.13±12.41 g/100 g of UPS that were not found significantly different than those noted in FPS that had total Ca content of 469.30±1.42 g/100 g. Whereas, the total Fe content in FPS (15.94±0.37 g/100 g) were found increased by ~1.05-fold as compared to those in UPS (15.15±0.38 g/100 g). Contrary to the Ca levels, Ca bioaccessibility in FPS (19.77 ±0.33%) was improved as compared to that observed in UPS (16.29 ±0.93%). Similarly, Fe bioaccessibility was considerably increased in FPS (16.32 ±0.39%) when compared with that of UPS (11.87 ±0.37%). The dialysis results showed a significant increase in the concentration of bioaccessibility of Fe implying an enhanced release and availability of this essential nutrient. The fermentation process appeared to play an important role in breaking down Ca binding compounds such as phytate and oxalate[22] and Fe-sequestering compounds, enhancing the bioavailability of both nutrients for absorption during digestion.

Table 2. Ca, Fe, and % bioaccessibility in unfermented and fermented perilla seeds

Formulation	Calcium (g/100 g)	Iron (g/100 g)
UPS	450.13±12.41[a]	15.15±0.38[b]
FPS	469.30±1.42[a]	15.94±0.37[a]

*UPS is unfermented perilla seeds and FPS is fermented perilla seeds. All data are shown as the mean ± standard deviation (SD) of triplicate determination (n = 3). Values with the same letter within the same column are not significantly different at $p < 0.05$.

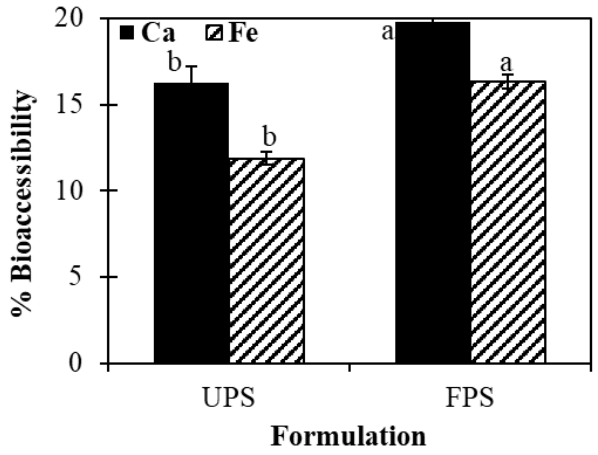

Figure 1. % Bioaccessibility of calcium and iron in unfermented (UPS) and fermented (FPS) perilla seeds. Data are reported as mean ± SD ($n = 3$) and alphabet indicate statistical difference ($p < 0.05$).

Simulated digestion and Caco-2 cell model studies to assess the bioavailability of Ca and Fe

We further investigated the mineral micelle fractions of UPS and FPS as presented in Table 3. The micelle fractions of Ca and Fe were found higher in the FPS, with values of 449.13±1.3 mg/100 g and 15.76±0.29 mg/100 g, respectively, compared to those of UPS that were 432.00±8.9 mg/100 g and 14.15±0.22 mg/100 g, respectively. Subsequently, the methods were diluted for preparation in Caco-2 cell testing. In the test medium, FPS showed higher calcium content (112.36±0.96 mg/100 g) compared to UPS (106.53±1.7 mg/100 g). Likewise, the Fe content in FPS (4.23±0.07 mg/100 g) surpassed that of UPS (3.72±0.11 mg/100 g). Uptake studies in Caco-2 cells demonstrated significant enhancement in both minerals' bioavailability when perilla seeds were exposed to fermentation (Figures 2a and 2b). Specifically, FPS showed a significantly higher Ca bioavailability compared to UPS, indicating a potential positive impact on absorption and utilization of this nutrient.

Figure 3 represents that the bioavailability of both Ca and Fe in FPS (22.44 and 17.11%, respectively) was significantly higher ($p < 0.05$) as compared to that of UPS (17.93 and 12.36%, respectively). These experiments suggested that the fermentation process has a positive impact on the bioavailability of dietary minerals in perilla seeds.

Table 3. Micelle fractions in unfermented and fermented perilla seeds

Mineral	Formulation	Micelle Fraction (mg/100 g)
Ca (mg/100g)	UPS	432.00±8.9[b]
	FPS	449.13±1.3[a]
Fe (mg/100g)	UPS	14.15±0.22[d]
	FPS	15.76±0.29[c]

*UPS = unfermented perilla seeds; FPS = fermented perilla seeds. Data are shown as the mean ± standard deviation (SD) of triplicate analysis ($n = 3$). Values with the same letter within the same column are not significantly different at $p < 0.05$.

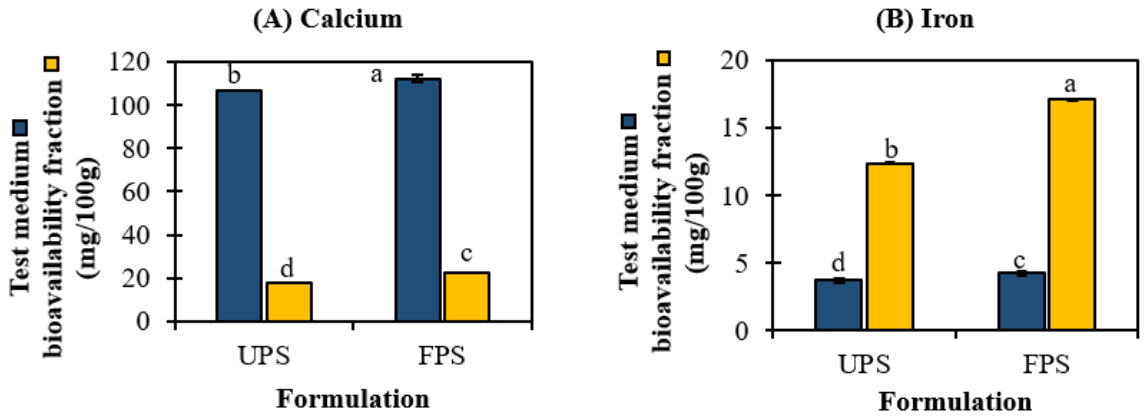

Figure 2. Test medium and bioavailability fractions of (A) Calcium and (B) Iron in unfermented (UPS) and fermented (FPS) perilla seeds. Data are reported as mean ± SD ($n = 3$) and alphabets indicate statistical difference ($p < 0.05$).

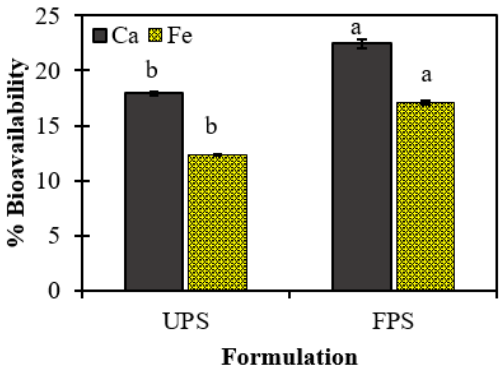

Figure 3. % Bioavailability of Ca and Fe in unfermented (UPS) and fermented (FPS) perilla seeds. Data are reported as mean ± SD ($n = 3$) and alphabets indicate statistical difference ($p < 0.05$).

Correlation between bioaccessibility and bioavailability

The correlation between the bioaccessibility and bioavailability of Ca and Fe in perilla seeds was established. Figures 4a and 4b represent the correlation curves for the percentage of Ca and Fe bioaccessibility and bioavailability, respectively. The significance of this section lies in its potential to demonstrate how effectively fermentation enhances the nutritional value of perilla seeds. By establishing a correlation between bioaccessibility (the release of nutrients from the seeds for absorption) and bioavailability (the proportion of nutrients absorbed and utilized by the body). Interestingly, the correlation strength varies between the two minerals. For Fe, a high R^2 value of 0.9486 indicates a very strong correlation between its bioaccessibility and bioavailability. This suggests that the bioavailability of Fe in perilla seeds is largely dependent on how accessible it is after the fermentation process. In contrast, the correlation for Ca bioaccessibility and bioavailability, is although strong, but not as notable as in case of Fe, implying different dynamics in how these minerals are processed by the body. The varying correlation strengths provide valuable information on nutrient absorption from FPS. A stronger correlation suggests that enhancing a nutrient's bioaccessibility through fermentation is likely to directly boost its bioavailability. This is particularly relevant for dietary planning, especially where addressing mineral deficiencies is a key concern.

The reasons behind the distinct R^2 values for Ca and Fe could stem from a variety of factors. These might include the inherent properties of the minerals, their interactions within the seed matrix, or the unique absorption mechanisms in the human body. A more in-depth analysis of the research, or additional sections, could provide further insights on these specific factors, to deepen our understanding of how fermentation changes the nutritional character of perilla seeds.

Discussion

Based on the hypothesis that fermentation mitigates the levels of antinutrients, this research attempted to expand existing knowledge regarding the biochemical alterations induced by fermentation in seeds and to inform potential applications in dietary regimes and food processing methodologies. In present study, the investigation of phytate and oxalate concentrations in perilla seeds showed a notable area of interest in the field of food science, particularly in terms of dietary nutritional bioavailability and health implications.

Phytic acid, also known as phytate or myo-inositol 1,2,3,4,5,6-hexakis-phosphate is the most common form of phosphorus storage, accounting for 1–5% of the content of cereals, legumes, oilseeds, and nuts[22]. Though, phytate possess antioxidative properties, it also has chelating property to be able to bind to minerals and make them inaccessible[16]. While phytate serves as an important source of phosphorus for plants, its prevalence in dietary sources can pose challenges for human nutrition owing to the ability to bind to essential minerals, such as Ca, Fe, and Zn, forming insoluble complexes that are poorly absorbed by the human body[23]. Consequently, high phytate intake has been associated with mineral deficiencies, particularly in populations with diets heavily reliant on grains and legumes. Similarly, oxalate is a naturally occurring compound found in various plant-based foods, including fruits, vegetables, grains, nuts, and seeds[24]. It forms oxalate salts when it binds with minerals like Ca, Mg, and K[25] and is also known to be related to the formation of kidney stones[17]. Fermentation processes have been known to affect the nutritional composition of seeds, potentially reducing the levels of anti-nutrients such as phytate, tannins, and polyphenols present in cereals [26]. Moreover, fermentation creates optimal pH conditions for enzymatic breakdown of phytate, which is found in cereals in complexes with multivalent cations like Fe, Zn, Ca, Mg, and proteins [14]. The reduction in phytate content results in a substantial increase in the solubility of Fe, Zn, and Ca by several times[27].

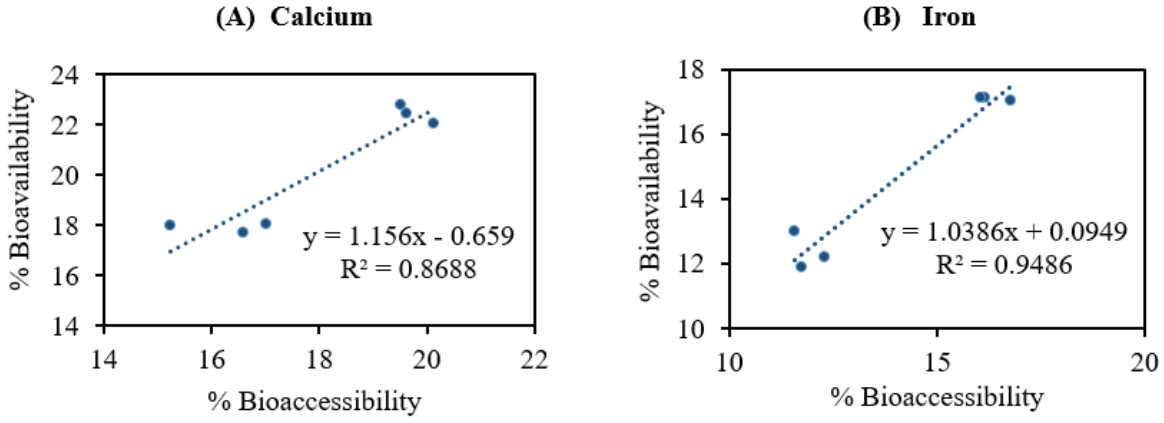

Figure 4. Bioaccessibility and bioavailability correlative curves of (A) Ca and (B) Fe in unfermented (UPS) and fermented (FPS) perilla seeds. Data are reported as mean ± SD (*n = 3*) and alphabets indicate statistical difference ($p < 0.05$).

The substantial decrease in phytate content observed in FPS in comparison to their unfermented counterparts suggests that the fermentation process effectively breaks down phytate. The results agree with the findings of Nkhata et al. (2018)[14], who showed that fermentation creates optimal pH conditions for the enzymatic breakdown of phytate. The reduction in phytate content observed in our study carries substantial nutritional implications. By decreasing phytate levels, fermentation may enhance the bioavailability of essential minerals, rendering them more readily absorbable within the digestive tract. Consequently, the consumption of FPS could potentially lead to improved mineral utilization, particularly among populations with dietary concerns related to phytate. The values of FPS found in this study are lower than those found in plants and cereals that have previously been found to have high phytate levels. The amount of phytate in soybeans varies from 1 to 2.2 g/100 g while that in mung beans is between 0.6 and 1.1 g/100 g[28]. There are several seeds and grains that have noticeable phytate levels, such as rice (1.1 g/100 g), cowpea (0.6 g/100 g), and soybean(0.9 g/100 g)[28].

Similarly, there was a significant reduction of the oxalate content in FPS as compared to that of UPS. Oxalates in foods exist in two forms: soluble and insoluble. Soluble oxalates include oxalic acid and its soluble salts, whereas insoluble oxalates are primarily composed of compounds like calcium oxalate. The method of extraction influences which form of oxalate is isolated. Significant observation was made regarding the extraction techniques for oxalates previously by Liu et al. (2015), focusing on the extraction and measurement of total and soluble oxalates in materials used for pulping and papermaking, [16]. This study reported that the application of hot distilled water was particularly effective in extracting soluble oxalates. On the other hand, the extraction of insoluble oxalates necessitated the use of an acid solution. The effectiveness of different extraction methods highlights the variable chemical properties of soluble and insoluble oxalates, and their varied responses to extraction solvents. In the present study, the extraction of oxalates from perilla seeds was performed using hot distilled water, which is proficient at extracting only soluble oxalates. This approach might explain why the results predominantly reflected changes in soluble oxalate levels. The insoluble oxalates, which would require an acid solution for extraction, remained unanalyzed. The findings from this study highlight the complexity of oxalate content in foods and the importance of considering both soluble and insoluble forms.

The fermentation process appears to alter the oxalate content in perilla seeds, but the extent and significance of this change may require more comprehensive research methods that account for both soluble and insoluble oxalates. However, the precise mechanisms underlying the microbial degradation of oxalate remain poorly understood at the molecular level. Furthermore, it is important to note that the degradation of oxalate and phytate during fermentation may result in increased bioavailability of essential minerals such as Ca and Fe. This increase is likely due to the breakdown of oxalates and phytates, which tend to form complexes with minerals, thereby diminishing their bio-availability.

Bioaccessibility is a term used in the field of nutrition and food science to describe the amount of bioactive compounds or nutrients that are available for absorption in the intestine after undergoing simulated *in vitro* gastrointestinal digestion [29]. Ca bioaccessibility represents the amount of Ca that could potentially be absorbed by the human body, contingent on the soluble Ca liberated from the food matrix during digestion. While the bioaccessibility of Fe can be enhanced by combining appropriate foods in the diet and reducing inhibitory factors [30]. However, plant-based diets rich in antinutrients like phytates and tannins can decrease the bioavailability of Fe [31]. Phytates, in particular, have a negative impact on Fe bioaccessibility [32].

Dialysis method or dialyzability assays, introduced in 1981 by Miller et al[21], provide a means to estimate Fe bioaccessibility from foods. This model, based on equilibrium dialysis, measures soluble minerals of low molecular weight. The process involves adding dialysis tubing with a specific molecular weight cut-off post-gastric digestion. The tubing, containing a buffer, sodium bicarbonate, diffuses slowly and neutralizes the peptic digest. After incubation, pancreatin/bile is added, and subsequent incubation allows the determination of total dialyzable Fe by measuring the mineral content in the dialysate. The premise of dialyzability methods lies in the assumption that dialyzable compounds are available for absorption in the small intestine. This method has been applied and slightly modified to study the bioaccessibility of various micronutrients, including Ca, Zn, and Mg, among others [33].

We reported presently that Ca and Fe bioaccessibility has significantly increased in FPS compared to their unfermented counterparts (as shown in Figure 1). Fermentation is known to increases mineral bioaccessibility, bioavailability, and digestibility, mostly by rupturing the tissues and cell walls of

plants and releasing enzymes and other bioactive substances [34]. Additionally, the food medium's lower pH values, which are obtained during fermentation, may help with mineral absorption as well as the reduction of phytate and oxalate that hinder mineral bioaccessibility [14].

Similarly, fermented cereals showed improved Fe and Zn bioaccessibility compared to non-fermented cereals. Household processing methods like soaking, cooking, germination, and fermentation have been found to reduce Fe binders and increase the bioavailability of these minerals in plant-based diets [35]. The availability of Fe in food has been found increased after fermentation mediated by lactic acid bacteria[36].

Human colon carcinoma cell line (Caco-2) is a validated intestinal epithelium model that displays most of the functional and structural characteristics of mature human enterocytes[20]. *In vitro* studies on mineral incorporation in Caco-2 cells, has improved bioavailability assays based on *in vitro* digestion and measurements of the soluble or dialyzable elements. In present study, significantly enhanced Ca and Fe bioavailability in FPS using Caco-2 cells adds valuable information to both nutritional science and food technology. Probiotics, or fermenting microorganisms, especially the lactic acid producing varieties, are primarily responsible for these health benefits[37]. Although other *Lactobacillus* cultures, like molds and yeast, are also utilized for fermentation, *Lactobacillus* and *Lactococcus* are the most commonly used[38]. The enhanced bioavailability of Ca and Fe in fermented perilla seeds aligns with previous studies emphasizing the positive impact of fermentation on mineral absorption.

Different fermentation processing techniques have demonstrated an ability to enhance the mineral bioavailability of plant-based foods. According to Bahaciu et al., (2018), five *Lactobacillus* strains had the potential to increase mineral bioavailability [39] and the phytate content are decreased by *L. fermentum B4655, L. plantarum B4495, L. casei B1922, L. bulgaricus CFR2028, and L. acidophilus B4496* strains of *Lactobacillus* when used for the process of soymilk fermentation at 37 °C for a 24 h period[40]. Furthermore, it has been demonstrated that the fermented soymilk has higher levels of Mg and Ca compared to the unfermented soymilk [41]. In the case of linseed, fermentation with *Lactobacillus acidophilus* has shown an increase in the bioavailability of Fe and Ca compared to raw seeds[42]. Similarly, fermented cereals showed improved Fe and Zn bioaccessibility compared to non-fermented cereals[43]. The mechanism behind the enhanced bioavailability appears to be linked to the optimal pH

conditions created during fermentation, facilitating the enzymatic breakdown of phytate.

The reduction in phytate content due to fermentation could result in a significant increase in the solubility of essential minerals multiple times over[27]. According to the report of Barakoti and Bains (2007), there might be a connection between increased Fe bioavailability in mung beans and a reduction in phytate content within fermented samples[44]. Hence, the comparative analyses of present study with the previous reports highlight the understanding on the aspects of mineral bioavailability and provides further insights into the knowledge about growth requirements of human body. Nevertheless, our study has certain limitations and further investigations are required on the aspects of strain improvement and examining specific strains of microorganisms involved in the fermentation process that lead to influence the mineral bioavailability. Additionally, investigating the long-term effects on consuming FPS on *in vivo* minerals' level would provide a more absolute understanding of their potential benefits. Practically, our findings suggest that FPS could serve as a valuable dietary source for individuals aiming to optimize their Ca and Fe intake. Furthermore, the improved bioavailability may hold particular significance for populations vulnerable to mineral deficiencies, such as those with malabsorption disorders or dietary restrictions.

The correlation between bioaccessibility and bioavailability in FPS, showed a detailed view of how nutrients work. Bioaccessibility is an important step prior to bioavailability that is about the amount of nutrients absorbed and utilized by the body. The relationship between these two concepts plays a big role in nutrition science, which determines the efficiency of food sources and processing techniques to deliver nutrients to the body.

In present research, the strong correlation of Fe bioaccessibility and bioavailability in FPS suggests that the bioavailability of Fe in these seeds is dependent on its bioaccessibility. This finding is noticeable as it highlights the effectiveness of fermentation in enhancing the release and subsequent absorption of Fe and evidently plays a pivotal role in making Fe more accessible and absorbable. This idea is similar to what other studies on fermented grains have found – they often show that minerals become more accessible after fermentation.

Furthermore, the correlation between bioavailability and bioaccessibility indicates the efficiency of body's absorption mechanisms. For example, a nutrient with high bioaccessibility (easily released during

digestion) might not be concomitantly bioavailable (poorly absorbed or utilized by the body), leading to poor mineral utilization. Taking Ca in perilla seeds as an example, its slightly lower R^2 value of 0.8688 shows a strong connection between bioaccessibility and bioavailability, indicating that the factors other than bioaccessibility, such as the person's gut health conditions, might affect as how well Ca is utilized.

The difference in the correlation strengths for Ca and Fe in this study underlines the complexity of nutrient absorption and utilization. It suggests that while fermentation significantly enhances the bioaccessibility of both minerals, their bioavailability is influenced by additional, possible nutrient specific factors such as, interaction with other chemical constituents present in the medium or physiological and metabolic conditions of the individual's body. This is a critical consideration for nutritionists and diet planners, especially in contexts of addressing mineral deficiencies. It implies that while fermentation can be an effective method to enhance nutrient availability, its impact might vary across different nutrients.

Overall, this study provides a wider knowledge of how methods to process food, such as fermentation, can improve nutritional value of food. By increasing the bioaccessibility of nutrients, fermentation could be a process used to improve the nutritional profile of plant-based foods. The correlations between bioaccessibility and bioavailability, and vice versa, in FPS provide valuable insights into the nutritional implications of fermentation by highlighting the importance of considering both the release and absorption of nutrients in dietary planning and food processing. The varying correlations for different minerals underscore the need for a nuanced approach to nutrition, recognizing that each nutrient may behave differently.

Conclusion

The study demonstrated that FPS are beneficial due to notably reduced phytate and oxalate contents, that was found associated to enhanced bioavailability and bioaccessibility of perilla seed minerals. The results suggested that the fermentation positively effects perilla seeds' nutritional value. The biological studies outlined in this work based on *in vitro* assays provide a basis for future studies. To gain further understanding of the pharmacological and nutritional value of FPS, the toxicological profile of active compounds especially the aromatic one and *in vivo* studies are required. This might lead to increased ethnobotanical uses of perilla to develop it further as plant-based dietary supplement,

functional food and nutraceutical to benefit human health by maintaining nutritional status especially in the people who are vegetarian or rarely consume meat.

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Conflicts of Interest

The authors declare no conflict of interest.

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