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# The effect of fortification on in vitro iron and zinc bioavailability in plant-based meat alternatives

Tugba Kose [a,d,e], Tessa de Bie [b], Renzi Wang [a], Ans Eilander [b], Anne J. Wanders [b], Paul A. Sharp [a,c,*]

[a] Department of Nutritional Sciences, School of Life Course and Population Sciences, King's College London, Franklin-Wilkins-Building, 150 Stamford Street, London SE1 9NH, UK
[b] Unilever Foods Innovation Centre, WH Wageningen 6708, the Netherlands
[c] School of Food Science & Nutrition, University of Leeds, LS2 9JT, UK
[d] Department of Nutrition and Dietetics, Faculty of Health Sciences, Hitit University, Çorum, Turkey
[e] Food Innovation and Health, Quadram Institute Bioscience, Norwich Research Park, Norwich NR4 7UQ, UK

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ABSTRACT

Plant-based diets are increasingly popular due to their perceived health benefits and positive environmental impact. However, there are concerns that long-term adherence to plant-based diets could result in nutritional deficiencies, especially for minerals found in abundance in animal foods. Therefore, plant-based meat substitutes may be a vehicle for fortification to provide a source of bioavailable minerals. This study investigated the iron (Fe), zinc (Zn) and calcium (Ca) content and bioavailability from unfortified and Fe and/or Zn fortified plant-based mince (PBM) compared with animal mince. Total and bioaccessible mineral levels in animal mince, and PBM were determined using microwave digestion and in vitro simulated gastrointestinal digestion, respectively. Mineral bioavailability was assessed by exposure of Caco-2 cells to the digested food samples and measuring mineral uptake into the cells using Inductively Coupled Plasma Mass Spectrometry (ICP-MS). Total phytate levels were measured to calculate the phytic acid:mineral molar ratio (PA:Fe, PA:Zn, PA:Ca) as indication of the mineral bioavailability. Fe bioavailability from animal mince was significantly greater than from unfortified PBM. Fortification of PBM with Fe reduced the PA:Fe molar ratio to below 10, and increased Fe bioavailability compared with the unfortified PBM. Total Fe uptake from fortified PBM was equivalent to that from animal mince. Following fortification of PBM with Zn, the PA:Zn ratio remained high (>14), and bioavailability was not enhanced compared with the unfortified PBM ($P > 0.1$). This study highlights that Fe-fortified PBM can improve Fe bioavailability and provide similar amounts of Fe compared to beef mince, whereas more research is needed for Zn fortification of PBM. Fortification can be a promising approach to reduce the phytic acid:mineral molar ratio to mitigate the inhibitory effect of PA on mineral bioavailability.

## 1. Introduction

In the past decade, an increasing number of countries have begun integrating sustainability into their food policies and nutrition education programs for healthy diet (Mazac et al., 2021). Food-Based Dietary Guidelines have emerged as pivotal tools for promoting sustainable practices, particularly in nutrition and environmental settings (Clark et al., 2020; Mazac et al., 2021). These guidelines advocate specific dietary recommendations, such as prioritizing plant-based diets, emphasizing consumption of seasonal and local foods, reducing food wastage, selecting fish from sustainable sources, and limiting intake of red and processed meats, highly processed foods, and sugar-sweetened beverages (Bechthold et al., 2018). Therefore, plant-based diets, including vegan or vegetarian diets, have gained popularity worldwide. In Europe, the prevalence of vegan and vegetarian diets has been estimated to range from 1 % to 10 %, although exact figures vary between countries (Klapp et al., 2022).

Plant-based diets are associated with potential health benefits due to the higher content of fibre, folic acid, vitamins C and E, and various phytochemicals, as well as a predominantly unsaturated lipid profile.

* Corresponding author at: Department of Nutritional Sciences, School of Life Course and Population Sciences, King's College London, Franklin-Wilkins-Building, 150 Stamford Street, London SE1 9NH, UK.
*E-mail address:* paul.a.sharp@kcl.ac.uk (P.A. Sharp).

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This could lead to a decreased risk of developing metabolic diseases, such as Type 2 diabetes and chronic heart diseases (Mullins and Arjmandi, 2021). However, eliminating or decreasing consumption of animal products from the diet also increases the risk of certain nutritional deficiencies for example for the minerals; iron (Fe), zinc (Zn) and calcium (Ca), and for long-chain omega-3 fatty acids, and vitamin B-12 (Neufingerl and Eilander, 2023; Rousseau et al., 2020).

Zn deficiency and Fe deficiency (ID) are widespread among populations that heavily rely on monotonous plant-based diets as their primary source of energy intake (Lowe et al., 2020). Vulnerable groups, such as children and women of reproductive age, are at heightened risk of these deficiencies due to their increased physiological demands. Approximately 17.3 % of the world's population is affected by Zn deficiency (Wessells and Brown, 2012), and in 2019, ID-induced anaemia was reported to affect 40 % of children and 30 % of women of reproductive age globally (WHO, 2012) whereas it is estimated that 20 % of women of reproductive age in the UK and US have Fe deficiency (Stevens et al., 2022). In the UK, data from the National Diet and Nutrition Survey (NDNS) indicate that between 20 % and 50 % of young children (1.5–3.5 years), girls (11–18 years), and women of childbearing age (19–49 years) have Fe intakes below the Lower Reference Nutrient Intake (LRNI) (Public Health England, 2020). Additionally, around 25 % of adolescents were reported to have Zn intakes below the LRNI (Public Health England, 2020). These findings underscore the importance of careful dietary planning and, when necessary, supplementation to mitigate the risks associated with micronutrient deficiencies in plant-based diets.

Plant-based diets contain primarily non-heme Fe which has much lower bioavailability (<10 %) compared with heme Fe (15–35 %), which is predominantly present in animal-based products (Hurrell and Egli, 2010). Also, plant-based diets are rich in phytic acid (PA) that is a chelator of divalent minerals such as Zn and Fe and limit their absorption. Ascorbic acid and citric acid are known enhancers of non-heme Fe absorption and are found in fruits and cruciferous vegetables (Lynch and Cook, 1980). Ascorbic acid facilitates the conversion of ferric Fe to ferrous Fe, which has a greater capacity for absorption (Hurrell and Egli, 2010; Lynch and Cook, 1980). In addition to Fe, a significant portion of dietary Zn comes from animal products, with meat contributing a substantial amount to Zn intake in UK diets according to the NDNS (Public Health England, 2020). When meat is removed from the diet in favour of plant-based alternatives, there is typically an increase in consumption of phytate-rich foods including legumes and whole grain cereals (Hemler and Hu, 2019). However, PA can inhibit the absorption of both Fe and Zn from these plant sources (Hunt, 2003). Therefore, while individuals following plant-based diets can obtain adequate amounts of minerals from plant products, their absorption is often limited by the presence of PA.

To meet sustainable dietary guidelines, food companies have developed a range of plant-based foods including plant-based meats. This strategic focus has prompted substantial investments in innovative plant-based alternatives that closely mimic traditional animal-derived products (Wickramasinghe et al., 2021). This trend has broadened the range and diversity of plant-based food options available in the market. Meat substitutes primarily utilize ingredients such as soybeans, and other legumes, wheat and mycoprotein (Mylan et al., 2023). These substitutes are designed to closely resemble traditional animal-derived products, allowing them to be manufactured into formats like sausages, burgers, and meatballs (Mylan et al., 2023; Wickramasinghe et al., 2021).

Ingredients used for meat substitutes such as soybeans, legumes, wheat and mycoprotein are sources of numerous minerals, such as Fe, Zn, Ca, magnesium (Mg) and potassium (K) (Public Health England, 2021). However, there is limited research comparing the mineral profiles and bioavailability of plant-based meat alternatives to their non-vegetarian counterparts, which could impact the mineral status of individuals avoiding meat. Therefore, this study aimed to investigate the impact of fortifying plant-based mince (PBM) on the content and bioavailability of Fe, Zn, and Ca, compared to animal mince. Specifically, the mineral and PA content, bioaccessibility, and bioavailability of Fe, Zn, and Ca in both PBM and animal mince were assessed. In addition, this study addressed the question whether mineral content, bioaccessibility and bioavailability of these minerals in PBM would deviate from that of whole soybeans. This approach offers new insights into the content and bioavailability of fortified plant-based meat alternatives compared to traditional meat, informing dietary choices for consumers.

## 2. Materials and methods

### 2.1. Reagents and chemicals

Unless otherwise stated, all the reagents and chemicals used in this study were purchased from Sigma-Aldrich Ltd. (Dorset, UK). Solutions of enzymes (pepsin and pancreatic-bile extract) were all freshly prepared just before use.

### 2.2. Sample collection and preparation

Animal minced meat (beef) was sourced from 4 different supermarkets in the Netherlands (Aldi, Albert Heijn, Lidl, and Jumbo). The animal minced meat was mixed (ratio of 1:1:1:1) and baked for 13 min, moisture loss from raw mince was 31.6 %. Soybeans ready for consumption from 3 different brands (HAK, Jumbo, Bonduelle) were mixed (ratio of 1:1:1), a kitchen sieve was used to remove moisture. PBM specifically designed for this study (based on soy and wheat protein) was sourced from Unilever and undergoing a heat treatment during the production process. The PBM provided was made with textured vegetable protein (extruded protein isolate and concentrate), consisting of 84 % soy protein and 12 % wheat protein. All PBM were provided with different fortification levels: an unfortified PBM, a PBM fortified with 2.1 mg/100 g ferric pyrophosphate, a PBM fortified with 1.5 mg/100 g zinc oxide and a PBM fortified with 2.1 mg/100 g ferric pyrophosphate and 1.5 mg/100 g zinc oxide. Products (100 g) were placed in an aluminium dish, frozen at −20 °C, and freeze-dried for five days using a freeze dryer (Alpha 1–4 L Dplus SciQuip Ltd, Newtown, UK). Dried animal mince and PBM were ground in a blender to obtain a fine powder, stored in sealed bags, and kept at −4 °C.

### 2.3. Determination of mineral content

The total mineral content of each dried product was determined using Perkin Elmer NexION 350D Inductively Coupled Plasma Quadrupole Mass Spectrometer (ICP-QMS) under Dynamic Reaction Cell mode with ammonia gas following microwave digestion, as outlined (Kose et al., 2019). Briefly, five independent 0.5 g aliquots of each food products were mixed with 10 mL of 70 % $HNO_3$. The tubes were then placed in a MARS 6 microwave digestion system (CEM Microwave Technology Ltd., Buckingham, UK) and heated to 210 °C for 45 min, followed by cooling to room temperature. For ICP-MS analysis, 250 μL of the digested products were diluted in tubes and adjusted to a final volume of 4.7 mL using Milli-Q water, with the addition of 50 μL of 5 ppm Gallium as an internal standard. Full details for instrument set-up and calibration curves for mineral analysis are shown in the Supplementary Information.

### 2.4. Phytic acid determination

The PA content (based on measurement of total phosphorus) in the food samples was determined using the Megazyme K-PHYT kit (Megazyme Ltd., Bray, Ireland), following the manufacturer's protocol described by McKie and McCleary (2016). In brief, acid extracts of 6 independent aliquots of each food products were treated with phytase to

degrade the inositol phosphates, and an alkaline phosphatase suspension was used to release phosphate from all forms of myo-inositol phosphates present. The total phosphate released was quantified using a modified colorimetric method. The results were expressed as grams of phosphorus per 100 g of dried sample material. The molar ratios of PA to minerals (Fe, Zn and Ca) were calculated as the millimole of PA present in the products divided by the millimole of minerals (Fe, Zn and Ca) present in the products, respectively. Molar ratios of PA to minerals that exceed specific thresholds are considered inadequate for absorption; PA: Fe ratio above 10 (Hurrell, 2004), PA:Zn ratio above 14 (WHO, 1996), and PA:Ca ratio above 17 (Umeta et al., 2005) are insufficient for Fe, Zn, and Ca absorption, respectively.

### 2.5. In vitro simulated gastrointestinal digestion

We followed an in vitro gastrointestinal digestion protocol established by Miller and colleagues in the 1980's (Miller et al., 1981) and subsequently modified by Glahn and colleagues in the 1990's (Glahn et al., 1998) to estimate iron absorption from foods. This protocol has been adapted and used extensively in our previous work to measure the release and uptake of minerals (including Fe, Zn and Ca) from foods (Aslam et al., 2024; Latunde-Dada et al., 2023). Briefly, 0.5 g products in the tubes were mixed with 10 mL of saline solution (140 mmol/L NaCl and 5 mmol/L KCl) and incubated for 5 min. Then, the pH was adjusted to 2.0, using 1 M HCl. Afterwards, 0.5 mL of pepsin (16 mg/mL) was added, and products were kept at 37 °C on a rotating platform (150 rpm) for 2 h. Afterwards, the pH of the samples was adjusted to pH 7 using 1 M $NaHCO_3$. Pancreatin-bile extract (2.5 mL containing 1.4 mg/mL pancreatin and 8.5 mg/mL bile) were added, the solution was made up to 20 mL with saline solution, and the products were incubated at 37 °C for 2 h. Two independent digests of each food product were carried out on separate days. At the end of the incubation digestion period, four independent aliquots from each food digest were taken to determine the total bioaccessible mineral fraction.

### 2.6. Determination of bioaccessible mineral fractions

Aqueous aliquots of the supernatants from digested products were centrifuged at 1000 rpm for 10 min to precipitate undigested food and to obtain the Total Bioaccessible Mineral (TBM) fraction. Afterwards, 200 μL of centrifuged samples were diluted in tubes and adjusted to a final volume of 4.75 mL using Milli-Q water, with the addition of 50 μL of 5 ppm Gallium as an internal standard. TBM minerals were measured by ICP-MS. Fe, Zn and Ca bioaccessibilities were measured as total mineral release (i) and fractional mineral release (ii) following in vitro digestion using the following equations.

i [Total mineral release (μM)] = [mineral content of digestate following centrifugation]

ii [Fractional mineral release (%)] = [mineral in digestate/initial mineral content of food sample] × 100

### 2.7. Cell culture

Human Caco-2 cell line was obtained from American Type Culture Collection (ATCC) at passage 19 and used in experiments at passages 27, 29, 33 and 35. Cells were sub-cultured in a 75 $cm^2$ flask to 70–80 % confluence. The growth medium contained Minimum Essential Medium (MEM), supplemented with 10 % foetal bovine serum (FBS), 1 % penicillin/streptomycin, 1 % antifungal/amphotericin B solution and 1 % MEM non-essential amino acids forming a complete media. The medium was changed every 2–3 days. Cells were kept at 37 °C under a humidified atmosphere containing 5 % $CO_2$.

### 2.8. Mineral bioavailability

The human Caco-2 cell line has been validated for use in the evaluation of bioavailability of minerals from different types of foods (Sandberg, 2010; Sharp, 2005). These cells are derived from colon adenocarcinoma and are employed as a surrogate for enterocytes in the small intestine. For experiments, Caco-2 cells were seeded in 6-well plates at a density of $1 \times 10^5$ cells/mL in complete media. The cells were allowed to differentiate over a period of 14 days, with the medium being changed every 2 days. Differentiation allows the cells to develop an enterocytes phenotype resembling cells found in the small intestine. Before starting the experiments, the cells in the 6-well plates were treated with 2 mL of serum-free medium (SFM) MEM (Minimum Essential Medium) for 24 h. This helps to remove any potential interference from serum components. Supernatants collected from the previously described digested food samples were heated at 100 °C for 5 min to inactivate digestive enzymes present in the supernatants and were allowed to cool. Digests were mixed 1:1 with SFM and added to Caco-2 for 4 h. Bioavailability measurements from each food sample were carried out using four separate passages of Caco-2 cells. For each passage, two wells of cells on separate plates were treated with the food digests, giving eight independent measurements of bioavailability in total for each food sample. At the end of the incubation period, cells were scraped from the plates and ICP-MS analysis of mineral uptake was performed as described (Kose et al., 2019). Measurements were made using a PerkinElmer NexION 5000 ICP-MS instrument which is optimized for routine multi-element analysis. Instrument operating conditions are detailed in Supplementary Table 1. The mineral (Fe, Zn and Ca) concentrations were calculated against the linear regression obtained from the calibration standard curve performed with each analysis (Supplementary Fig. 1). The method parameters used for the analysis of minerals; Fe, Zn and Ca by ICP-MS are provided in Supplementary Table 2. Fe, Zn and Ca bioavailabilities were measured as total mineral absorption (iii) and fractional mineral absorption (iv) by Caco-2 cells using the following equations.

iii [Total mineral absorption (nmol/mg protein)] = [mineral in cell lysate/protein content of cell lysate]

iv [Fractional mineral absorption (%)] = (mineral in cell lysate/mineral content of food sample] × 100

### 2.9. Statistical analysis

Statistical analysis was performed using GraphPad Prism 10.0 (GraphPad Software, USA) with one-way analysis of variance (ANOVA) and Tukey's multiple comparisons post-hoc test to compare the means of the experimental groups. This aligns with statistical approaches used in our previously published work using in vitro digestion models (Aslam et al., 2024; Latunde-Dada et al., 2023). In addition, a two-way ANOVA was conducted using R Statistical Software (Core Team, 2022) to examine the effects of Fe and Zn content, and their interaction in the PBM on respectively fractional Fe release and absorption and, fractional Zn release and absorption. Outliers were removed from the analysis when deviating more than 3 standard deviations from the mean. Normality was visually assessed using QQplots. Results are presented as mean ± standard error of the mean (SEM), and statistical significance was set at $P < 0.05$ for comparisons between groups.

## 3. Results

### 3.1. Mineral composition of animal mince, soybeans, and PBM

Levels of the minerals; Fe, Zn and Ca in the animal mince, soybeans indicated significant differences in mineral contents (Table 1). Total Fe content was significantly higher in PBM fortified with Fe and Fe+Zn compared to animal mince. Both soybean and unfortified PBM showed Fe levels comparable to animal mince. The Zn content in animal mince was significantly higher compared to all plant-based products of which unfortified PBM showed the lowest. PBM fortified with Zn and Fe+Zn contained double amount of Zn compared to the unfortified PBM. Ca

**Table 1**
Mineral and moisture content of animal mince, soybeans, and plant-based meat (PBM) (per 100 g dry weight).

| Sample | Fe (mg/100 g) | Zn (mg/100 g) | Ca (mg/100 g) | Moisture content (%) |
|---|---|---|---|---|
| **Animal mince** | $8.7 \pm 0.8^{a}$ | $14.7 \pm 0.4^{a}$ | $31.7 \pm 1.4^{a}$ | 48.3 |
| **Soybeans** | $9.5 \pm 0.5^{a}$ | $7.7 \pm 0.3^{b}$ | $440.1 \pm 26.6^{b}$ | 73.5 |
| **PBM** | $9.5 \pm 0.5^{a}$ | $4.6 \pm 0.1^{c}$ | $309.9 \pm 17.5^{c}$ | 69.6 |
| **PBM +Fe** | $15.8 \pm 0.8^{b}$ | $4.7 \pm 0.5^{c}$ | $286.8 \pm 19.8^{c}$ | 67.5 |
| **PBM +Zn** | $8.6 \pm 0.1^{a}$ | $9.8 \pm 0.04^{d}$ | $277.8 \pm 17.1^{c}$ | 69.1 |
| **PBM+Fe+Zn** | $16.4 \pm 0.5^{b}$ | $10.2 \pm 0.7^{d}$ | $300.9 \pm 7.5^{c}$ | 69.8 |

concentrations in all plant-based products were significantly higher compared to the animal mince.

Data are presented as mean + SEM (Standard Error of the Mean) from triplicate measurements. Groups not sharing common letters are statistically different ($P < 0.05$), one-way ANOVA, and Tukey's post-hoc test. PBM, Plant-based mince; PBM +Fe, Plant-based mince fortified with iron; PBM +Zn, Plant-based mince fortified with zinc; PBM +Fe+Zn, Plant-based mince fortified with iron and zinc.

### 3.2. Bioaccessibility of animal mince, soybeans, and PBM

Following *in vitro*-simulated gastrointestinal digestion, the total release of minerals was evaluated using ICP-MS (Fig. 1A, C). Derived from this analysis was the fractional mineral release expressed as a

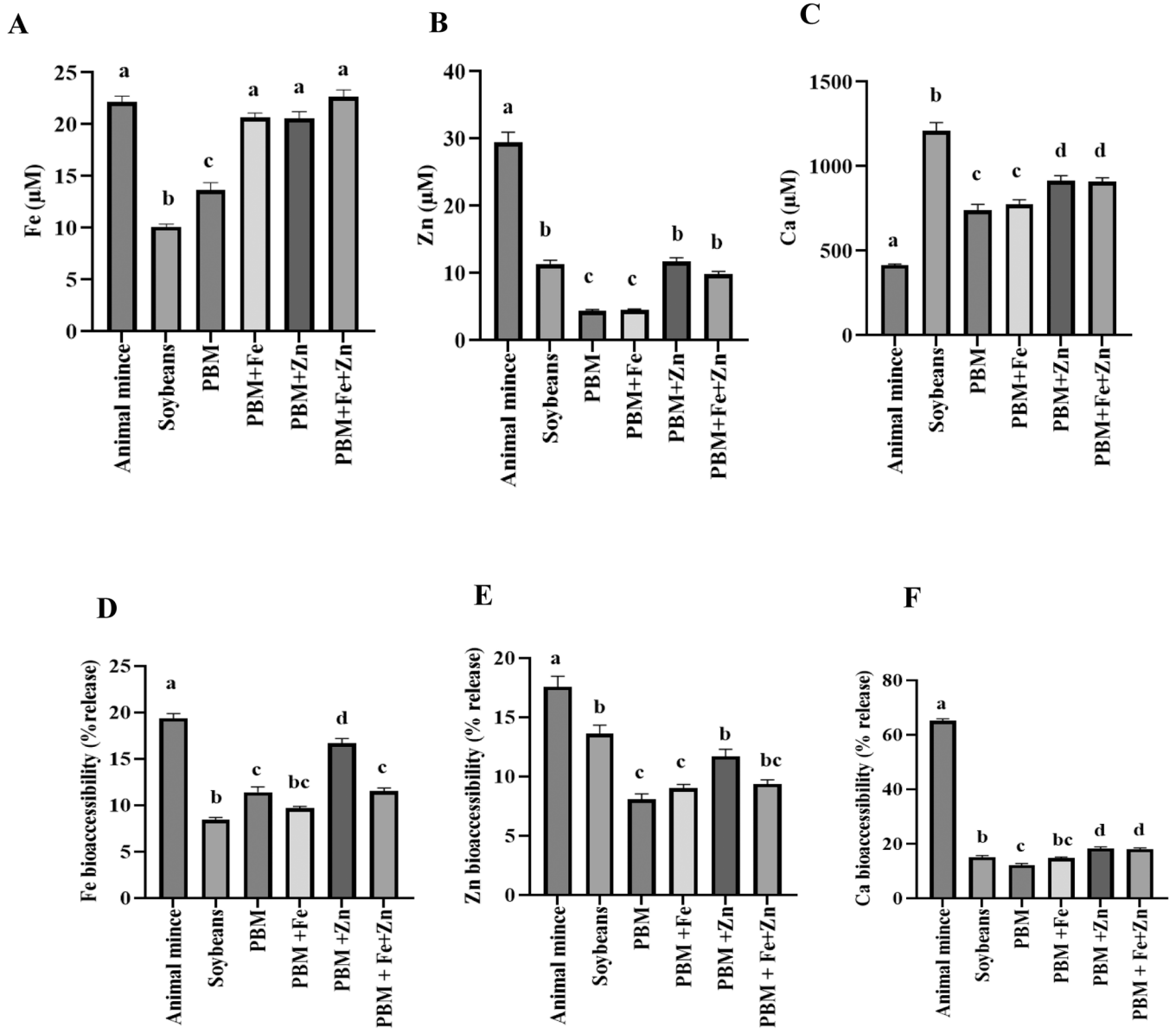


**Fig. 1.** Bioaccessible minerals in animal mince, soybeans, and PBM. Total Fe (A), Zn (B) and Ca (C) release measured as total mineral contents (µM) released from dry food following in vitro digestion. Fractional Fe (D), Zn (E) and Ca (F) release measured as % of the initial mineral contents released from dry food following in vitro digestion. Data are presented as the mean ± SEM (Standard Error of the Mean), n = 8. Groups not sharing common letters are statistically different (P < 0.05), one-way ANOVA and Tukey's post-hoc test.

percentage of the mineral content in the original dry products (Fig. 1D-F). Animal mince had the highest total and fractional Fe release compared to non-fortified PBM and soybeans. Total Fe release was similar in fortified PBM to that in animal mince. Among PBM, those fortified with Fe, Zn, or Fe+Zn demonstrated higher total Fe release than non-fortified PBM. However, fractional Fe release in PBM fortified with Fe or Fe+Zn was similar to that in non-fortified PBM (Fig. 1A, D). PBM fortified with Zn had a significantly higher fractional Fe release as compared to non-fortified PBM. Indeed, among the PBM there was a significant interaction between Fe and Zn content on the Fe release ($p = .0006$). The addition of Zn increased the Fe release especially in the PBM fortified with only Zn.

For Zn, animal mince also exhibited the highest levels of total and fractional release following *in vitro* digestion (Fig. 1B, E). PBM fortified with Zn or Zn+Fe showed higher total Zn release than non-fortified PBM, but fractional Zn bioaccessibility was significantly higher only in PBM fortified with Zn (Fig. 1B, E). There was also a significant interaction between the Zn and Fe content on the Zn release ($p = .001$), showing that the addition of Fe blunted the effect of Zn addition on Zn release. Regarding Ca, all PBM released more total Ca compared to animal mince (Fig. 1C). However, the fractional Ca release was highest in animal mince (where total Ca content was low) compared to the PBM (Fig. 1F). Among the PBM, Ca release and fractional release were higher in the PBM fortified with Zn.

### 3.3. Mineral bioavailability from animal mince, soybeans, and PBM

To estimate the mineral bioavailability of the products, the *in vitro* simulated gastrointestinal digestion was followed by the assessment of mineral uptake in Caco-2 cells. Total and fractional mineral absorption (i.e. uptake by Caco-2 cells) were assessed (Fig. 2). Animal mince and PBM fortified with Fe led to a similar uptake of Fe and showed higher Fe uptake than the other products. However, animal mince had a higher fractional Fe absorption than other tested products (Fig. 2A, D). Interestingly, the Zn fortification of PBM led to a higher fractional absorption of the inherent Fe, while the combination of both Fe and Zn fortification led to a lower fractional absorption of Fe than foods fortified with Fe or Zn alone. Statistical analysis also showed a significant interaction effect

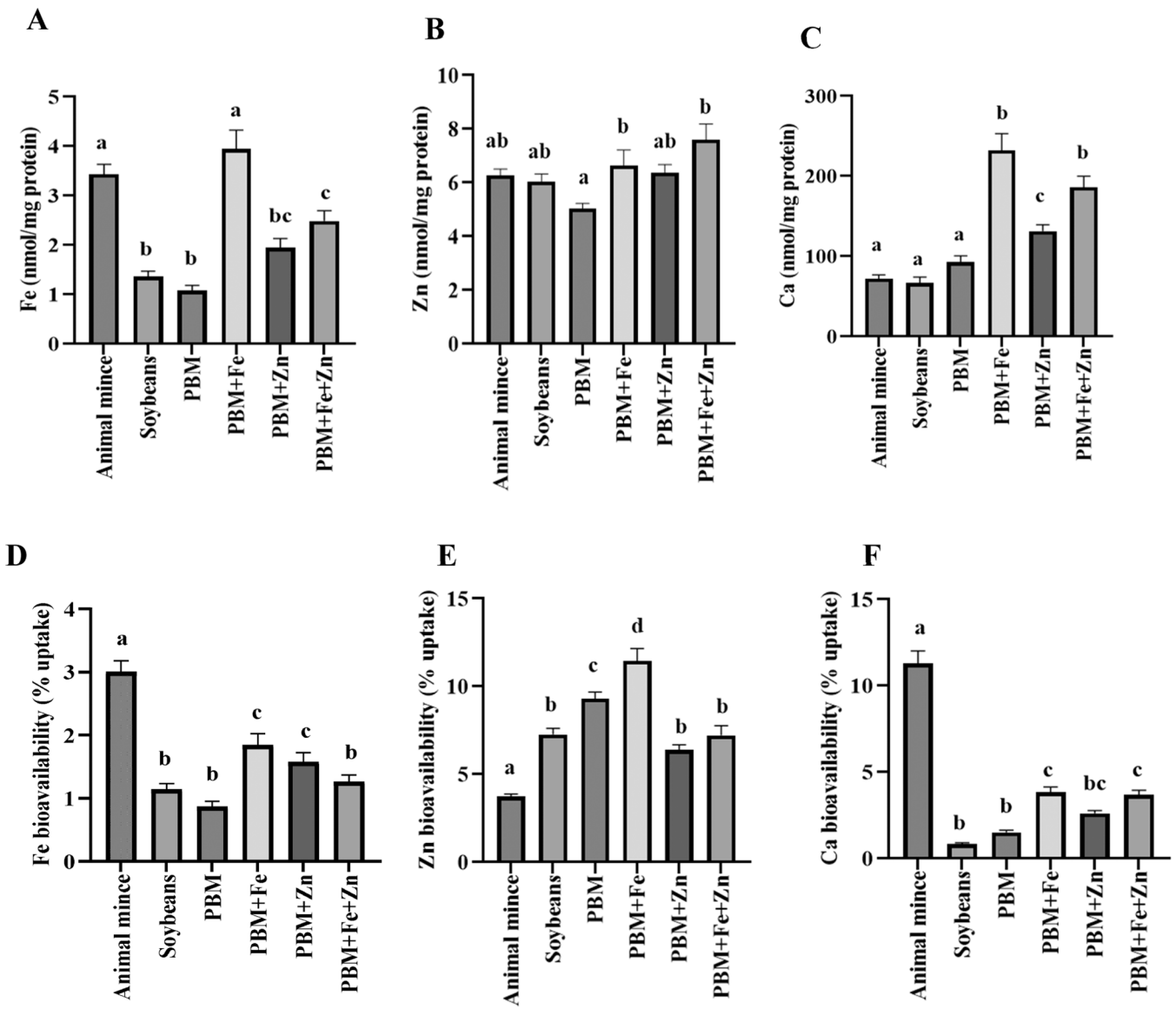


**Fig. 2.** Mineral bioavailability from animal mince, soybeans, and PBM. Total Fe (A), Zn (B) and Ca (C) absorption, measured as the absolute mineral (μM) taken up by Caco-2 cells from dry food following in vitro digestion. Fractional Fe (D), Zn (E) and Ca (F) absorption, measured as % of the initial mineral contents, taken up by Caco-2 cells from dry food following in vitro digestion. Data are presented as the mean ± SEM (Standard Error of the Mean), n = 8. Groups not sharing common letters are statistically different ($P < 0.05$), one-way ANOVA, and Tukey's post-hoc test.

of Zn and Fe fortification on the fractional Fe absorption (p = .004).

In terms of the Zn profile of the samples, total Zn uptake was not different between animal mince, PBM and soybeans (Fig. 2B). PBM fortified with Fe and Fe+Zn had a higher total Zn uptake than non-fortified PBM. As compared to animal mince and soybeans the fractional Zn absorption was significantly higher in PBM (Fig. 2E). This further increased with Fe fortification while the addition of Zn led to a significantly lower fractional absorption. This was also confirmed by a significant interaction between Fe and Zn content on the fractional Zn absorption (p = .05).

For Ca, soybeans and PBM had a significantly higher content than animal mince (Table 1). Despite that the fractional absorption of Ca from animal mince was highest, PBM demonstrated similar total Ca uptake (Fig. 2C, F). Fortification with Fe and Fe+Zn increased the total and fractional absorption of Ca compared to unfortified PBM.

### 3.4. Phytic acid levels in animal mince, soybeans, and PBM

Table 2 shows PA content, and the molar ratios, PA:Fe, PA:Zn, and PA:Ca. The highest PA:Fe ratios were for soybeans, PBM and PBM+Zn, indicating that Fe absorption from these foods might be significantly inhibited by phytate content. PBM and PBM fortified with Fe exhibited the highest PA:Zn molar ratio, whereas Zn fortification reduced this ratio in PBM fortified with Zn and Fe+Zn. Regarding the molar ratio of PA:Ca, animal mince had the highest value compared to other products; however, this finding can be misleading. This is due to the low levels of Ca and PA present in animal meat, which results in a high ratio that does not accurately reflect the true relationship between these components.

Data are presented as mean ± SEM (Standard Error of the Mean) from triplicate measurements. Groups not sharing common letters are statistically different ($P < 0.05$), one-way ANOVA and Tukey's post-hoc test. PBM, Plant-based mince; BMI, PBM +Fe, Plant-based mince fortified with iron; PBM +Zn, Plant-based mince fortified with zinc; PBM +Fe+Zn, Plant-based mince fortified with iron and zinc; PA:Fe, the molar ratios of Phytic acid-to-Iron; PA:Zn, the molar ratios of Phytic acid-to-Zinc; PA:Ca, the molar ratios of Phytic acid-to-Calcium.

## 4. Discussion

The current study aimed to assess the content and availability of Fe, Zn, and Ca from animal mince and PBM. The findings suggest that fortifying plant-based meat products with Fe and/or Zn could significantly enhance their nutritional value and potentially is an effective strategy to prevent mineral inadequacies in the transition towards more plant-based diets.

The consumption of plant-based products is expanding by 15 % per annum in the UK and thus there is an increasing interest in providing plant-based meat substitutes with enhanced nutritional quality to the market (Hemler and Hu, 2019). The current study shows that in PBM Fe content was similar and Ca content was higher when compared to animal mince, whereas total Zn content was lower, even when PBM was fortified with Zn. These observations are in line with available data on beef, plant-based meats and the key ingredients of plant-based meats. Food composition data indicate that soy and other legumes are typically rich in Fe and Ca, but lower in Zn; that wheat has moderate levels of Fe and Zn and is lower in Ca, whereas beef is rich in Zn, moderate in Fe and low in Ca (Public Health England, 2021). A recent study analysing various plant-based burgers in the UK market, found that an iron-fortified soy-based burger had 14.5 mg Fe per 100 g dry weight and a beef burger had 3.4 mg Fe per 100 g dry weight (Latunde-Dada et al., 2023). In another study, Swing et al. (2021) analysed soy-based and pea-based burgers and found that fortified plant-based burgers had double the amount of total Fe (3.63 mg/100 g fresh weight) compared to animal-based beef burgers (1.94 mg/100 g fresh weight). Similarly, Harnack et al. observed lower total Zn content and higher Ca in plant-based alternatives than in ground beef (Harnack et al., 2021). Variations of mineral levels in plant-based meat can be primarily attributed to differences in protein sources and amounts of these sources used in meat alternatives, fortification levels, but can also be attributed to the processing methods, variations in product development practices, and the inclusion of wheat flour components, which is mandatorily fortified with Fe in the UK (Costa-Catala et al., 2023).

Bioaccessibility is a measure of mineral release from food during digestion whereas bioavailability is a measure of the amount of the released mineral that is absorbed by the intestine and either stored within the body or utilised for metabolic purposes (Arafsha et al., 2023). The current study showed that Fe release and absorption were both lower from PBM than from animal mince. However, fortification with Fe partially mitigated the reduced absorption from PBM.

Soy has previously been reported to have low Fe bioaccessibility, due to the high levels of PA (Erdman and Fordyce, 1989). In agreement with this, the current study shows a PA:Fe molar ratio of greater than 10 in unfortified PBM. However, Fe fortification reduced the PA:Fe molar ratio to below 10, a level generally considered to be adequate for Fe absorption (Hurrell, 2004), which resulted an increased fractional Fe absorption from PBM. A study by Taleon et al. (2020) supports this finding, showing that brown rice fortified with Fe+Zn improved mineral uptake by decreasing the PA:Fe and PA:Zn molar ratios. This suggests that fortification of foods in plant-based diets may help to achieve an adequate Fe intake.

Animal meat is the main source of Zn in UK omnivorous diets (Hunt, 2003). This study found that fractional Zn release from animal mince was significantly higher compared to PBM. Consistent with this finding, previous work has also shown that Zn bioaccessibility from beef burgers was significantly greater than from various meat substitute burgers made from soy, mycoprotein, mushrooms, potato or beetroot (Latunde-Dada et al., 2023). Surprisingly, despite the higher levels of PA and PA:Zn molar ratios in PBM, the fractional absorption of Zn was lower from animal mince compared to PBM, resulting in a similar total Zn uptake. It is possible that the Zn oxide used to fortify the PBM remained in a highly bioavailable form, but further research is needed to confirm and explain these observations. Moreover, the results from this study require validation in robust dietary intervention trials with human volunteers.

Although fractional Ca release and fractional Ca absorption were both higher from animal mince than from PBM, the 10 times higher Ca content in PBM resulted in higher total Ca absorption from PBM. A key factor is the inhibitory effect of PA, which was highly present in soybeans and PBM, on absorption due to the formation of low-solubility Ca-PA complexes in the intestinal lumen (Hunt, 2003). In contrast, the low PA content in animal mince may result in greater Ca solubility, leading to the highest fractional Ca absorption.

Given the high prevalence of ID anaemia and Zn deficiency in various global populations, combined fortification with Fe and Zn is considered to be a strategy to overcome the low bioavailability of these minerals from plant-based diets. However, human studies have shown that in supplementation trials both minerals can inhibit each other's absorption, indicating that it might be challenging to prevent mineral deficiencies through combined fortification (Christian et al., 2003a,b; Kolsteren et al., 1999; Zavaleta et al., 2000). The findings in this study

**Table 2**
PA content (g/100 g dry weight) and phytic acid:minerals molar ratio of the animal mince, soybeans, and plant-based meat (PBM).

| Samples | Phytic acid (g/100 g dry weight) | PA:Fe | PA:Zn | PA:Ca |
|---|---|---|---|---|
| **Animal mince** | $0.3 \pm 0.03^{a}$ | $2.7 \pm 0.5^{a}$ | $1.8 \pm 0.3^{a}$ | $0.5 \pm 0.1^{a}$ |
| **Soybeans** | $1.4 \pm 0.01^{b}$ | $12.3 \pm 0.8^{b}$ | $17.6 \pm 0.9^{b}$ | $0.2 \pm 0.02^{b}$ |
| **PBM** | $1.4 \pm 0.01^{b}$ | $12.2 \pm 0.8^{b}$ | $28.2 \pm 2.4^{c}$ | $0.3 \pm 0.03^{b}$ |
| **PBM +Fe** | $1.4 \pm 0.01^{b}$ | $7.2 \pm 0.7^{c}$ | $30.4 \pm 2.9^{c}$ | $0.3 \pm 0.3^{b}$ |
| **PBM +Zn** | $1.4 \pm 0.01^{b}$ | $12.7 \pm 1.3^{b}$ | $14.3 \pm 0.1^{b}$ | $0.3 \pm 0.02^{b}$ |
| **PBM +Fe+Zn** | $1.6 \pm 0.01^{c}$ | $8.1 \pm 0.3^{c}$ | $15.2 \pm 0.8^{b}$ | $0.3 \pm 0.01^{b}$ |

also indicated interactions: the addition of Zn increases the bioavailability of the endogenous Fe present in PBM, and similarly the addition of Fe seems to increase the bioavailability of endogenous Zn. The mechanisms underlying the interaction between Zn and Fe are not fully understood, although some hypotheses have been proposed (Kondaiah et al., 2019). One hypothesis is that Zn and Fe compete for a common absorptive pathway, potentially explained by competitive binding to the divalent metal transporter 1 (DMT1), a proton-coupled transporter for various divalent metals (Gunshin et al., 1997). Regarding Fe absorption, a study by Dijkhuizen et al. (2001) indicated that Fe supplementation alone was more effective in reducing anaemia in Fe-deficient infants, achieving a 38 % reduction compared to a 20 % reduction with combined Fe and Zn supplementation. Similarly, a Mexican trial, hemoglobin concentrations increased to 14.0 g/L in the Fe supplementation alone group and 13.0 g/L in the Fe+Zn supplementation group, though it is worth noting that in both trial arms hemoglobin was significantly higher than the 8.0 g/L in the placebo group (Muñoz et al., 2000). These results reflect the antagonistic effects of these micronutrients, consistent with our findings. Another hypothesis of the competitive interaction is the dose-dependent effect of these minerals on the intestinal absorption (Moradveisi et al., 2019). Studies suggest that the threshold for inhibitory effect on Fe absorption could occur at a Zn to Fe molar ratio of 5:1 (Olivares et al., 2007). Further research is needed to fully understand the interactions between Fe and Zn, particularly from foods fortified with these minerals. Regarding Zn absorption, there are conflicting data on the interactions between Fe and Zn. Some studies have reported that co-supplementation with Fe and Zn does not significantly affect Zn absorption; however, this effect may depend on the molar ratio of Fe to Zn (2:1) and the duration of the study (Sandström, 2001). In the present study, combined fortification did not affect Zn absorption in PBM compared to PBM fortified with Zn only.

A limitation of the current study is that Caco-2 cell model do not fully reflect the complex physiological responses seen in the intestine of living organisms. However, Caco-2 cells are widely used *in vitro* models for studying intestinal absorption and bioavailability of nutrients, because the cells express intestinal microvilli, and many of the transporters, enzymes and differentiation markers that are typically found in human small intestine enterocytes (Sharp, 2005). Additionally, the absence of systemic factors that influence mineral absorption may complicate the extrapolation of findings to *in vivo* studies (Miller and Berner, 1989). Nonetheless, evaluating the PA content, bioaccessibility and bioavailability of minerals in Caco-2 cells is valuable for predicting the relative bioavailability of various plant foods. These data can help to rank the bioavailability from a range of similar foods and identify those specific food products that would warrant further evaluation in human dietary interventions aimed at measuring the mineral bioavailability from meals that include meat substitutes.

## 5. Conclusion

In conclusion, fortifying PBM with Fe and Zn enhanced Fe, Zn, and Ca uptake. Fortification of PBM with Fe can improve Fe bioavailability and provide similar amounts of Fe compared to animal mince. However, the addition of Zn to PBM to enhance Zn absorption requires further study. As the population increasingly adopts plant-based diets, developing strategies to enhance mineral bioavailability will be of crucial importance to maintain nutrient adequacy. Fortification of plant-based meat can be an effective strategy to lower the phytate-to-mineral molar ratio, helping to reduce the inhibitory effect of phytate on mineral bioavailability.

## Author contribution

Conception and design of study: P.A. Sharp, T. Kose. Acquisition of data: T. Kose, R. Wang. Analysis and/or interpretation of data: P.A. Sharp, T. Kose, T. de Bie, A. Eilander, A. Wanders. Drafting the manuscript: T. Kose, T. de Bie. Revising the manuscript critically for important intellectual content: P.A. Sharp, T. de Bie, A. Eilander, A. Wanders. Approval of the version of the manuscript to be published: P.A. Sharp.

## CRediT authorship contribution statement

**Ans Eilander:** Resources. **Tugba Kose:** Writing – original draft, Software, Resources. **Renzi Wang:** Methodology. **Sharp Paul:** Writing – review & editing, Supervision, Resources, Funding acquisition. **Anne J. Wanders:** Resources. **Tessa de Bie:** Resources.

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## Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Paul A. Sharp reports financial support was provided by Unilever Innovation Centre Wageningen BV. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jfca.2025.107951.

## Data Availability

Data will be made available on request.

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