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Food–gut continuum of biofortified micronutrients: Influence of breadmaking processes on iodine, selenium, and zinc bioaccessibility and epithelial responses in an *in vitro* intestinal model☆

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ABSTRACT

Micronutrient malnutrition affects over 3 billion people worldwide. This study evaluated biofortified wheat breads with iodine, selenium, and zinc, applied individually or in combination, and examined mineral retention, bioaccessibility, and intestinal epithelial responses. Mineral concentrations in wheat and their changes after baking unfermented flatbread and fermented sourdough were quantified, while intestinal bioaccessibility and epithelial effects were assessed using *in-vitro* digestion and cell culture models. Biofortification significantly increased mineral concentrations in wheat; however, retention during baking varied by mineral and product. Flour composition and baking method influenced iodine and zinc levels, whereas selenium retention was primarily affected by wheat cultivar and fermentation. In biofortified Bezostaja-1, Se bioaccessibility reached 68% in flatbread, while iodine and zinc reached 49% and 12% in sourdough. Selenium enhanced mitochondrial activity in intestinal cells in both bread types, and zinc-enriched sourdough increased epithelial integrity by 15% and reduced cellular permeability by 30%.

1. Introduction

The adequate intake of essential micronutrients is fundamental to human health, well-being, and sustainable development (Ashraf, 2025; UNICEF, 2020). Recent studies document a transition in the prevalence and distribution of micronutrient deficiencies worldwide, with iodine insufficiency re-emerging in high-income regions such as parts of Europe. This shift is largely driven by changes in dietary patterns, particularly the rise of plant-based diets and reduced consumption of iodized salt, challenging long-standing public health assumptions (Gapp et al., 2024; Rigutto-Farebrother & Zimmermann, 2024; WHO, 2024). Selenium (Se) deficiency was traditionally associated with regions of low soil selenium, including central China, Eastern Europe, and sub-Saharan Africa, emerging pressures from soil degradation, intensive agriculture, and climate change are increasing the risk of deficiency across broader geographic regions (Oumer et al., 2024; Rayman, 2012). Moreover, zinc (Zn) continues to be one of the most widespread micronutrient inadequacies globally, particularly in cereal-based diets

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and among older adults and individuals with chronic illnesses (Gupta et al., 2020; Passarelli et al., 2024).

To address these challenges, biofortification, the increase of nutritional content of food crops through agronomic practices, conventional breeding, or genetic approaches, has emerged as an evidence-based approach to mitigate micronutrient deficiencies sustainably (Bouis & Saltzman, 2017). Although progress has been made in the development of cereals biofortified with single nutrients, simultaneous enrichment of staple grains with multiple minerals presents distinct strategic advantages. Multi-nutrient (MN) biofortification increases the nutritional density of staple foods without requiring changes in consumer behaviour. Consistent with this, field-based studies have demonstrated that MN biofortification effectively enhances Zn, I, and Se concentrations in cereal grains such as wheat and rice (Prom-u-thai et al., 2020; Ram et al., 2024; Zou et al., 2019). However, possible nutrient–nutrient interactions, such as competitive uptake or translocation antagonism, must be carefully identified and managed. For example, in a field study on wheat conducted in Yangling, China, the foliar co-application of zinc sulfate ($ZnSO_4$) and sodium selenite resulted in a 32–37% reduction in grain Se concentration and estimated daily Se intake compared to treatments with Se alone (Ning et al., 2022). In a multi-country wheat study involving large-scale field trials, a foliar spray containing zinc sulfate heptahydrate ($ZnSO_4{\cdot}7H_2O$, 0.5%), potassium iodate (KIO_3, 0.05%), sodium selenate (Na_2SeO_4, 0.001%), and iron ethylenediaminetetraacetic acid (Fe-EDTA, 0.02%) was applied twice during the growing season. The intervention led to an increase in grain concentrations: 28.6 to 46.1 mg/kg for Zn, 24.4 to 361.0 μg/kg for I, and 93.1 to 325.1 μg/kg for Se, along with an average increase of approximately 12% in grain iron (Fe) concentration. No negative interactions among the nutrients were observed (Zou et al., 2019). Similarly, Prom-u-thai et al. (2020) reported that foliar sprays of 0.5% $ZnSO_4$ and 0.05% KIO_3, when applied alongside Fe and Se in irrigated rice systems, enhanced grain concentrations of all target micronutrients, confirming the feasibility of Zn and I co-application within MN biofortification protocols.

Beyond the agronomic performance, the ultimate efficiency of these strategies depends on the metabolic fate of micronutrients within the human gastrointestinal tract. A distinction exists between the total mineral concentration in a crop and its gastrointestinal release and bioaccessibility, defined as the fraction of the nutrient released from the food matrix during gastrointestinal digestion and rendered available for absorption. Mineral release and bioaccessibility can vary with intrinsic crop characteristics, including genotype-dependent differences in mineral distribution and interactions with the food matrix. For instance, Lončarić et al. (2021) showed that Se and Zn co-biofortification in wheat increases both total concentrations and bioaccessible fractions, while the efficiency of nutrient release remains genotype-dependent. Moreover, post-harvest processing further modulates bioaccessibility by altering food structure and reducing antinutrients such as phytates through milling, fermentation, or thermal treatment.

However, bioaccessibility alone does not necessarily translate into nutritional benefit. The physiological efficacy of these minerals depends on the integrity and functional capacity of the intestinal epithelial barrier, which serves as a selective gatekeeper. Iodine, Se, and Zn play distinct roles in maintaining mucosal homeostasis, and their uptake requires functional epithelial transport systems. Zinc is absorbed primarily *via* the ZIP (SLC39) family for influx and the ZnT (SLC30) family for intracellular trafficking and basolateral efflux. Selenium uptake occurs mainly as selenoamino acids through amino acid transporters, whereas inorganic selenate and selenite follow distinct carrier-mediated or diffusion pathways. Iodine is absorbed efficiently as iodide through rapid passive and carrier-mediated processes, while the sodium–iodide symporter is largely restricted to thyroidal uptake. Efficient absorption therefore depends on both nutrient availability and transporter functionality.

Once internalized, these micronutrients exert essential cellular effects. Zinc is critical for tight junction assembly, specifically zonula occludens-1 and occludin, which seal the paracellular space and prevent the translocation of luminal pathogens. Concurrently, Se functions through the synthesis of selenoproteins, such as glutathione peroxidase 2, which provides antioxidant defense for the intestinal mucosa by neutralizing reactive oxygen species and preventing oxidative damage to enterocytes (Shao et al., 2017). Iodine further complements this system by influencing thyroid-dependent metabolic signalling, which is essential for the rapid turnover and differentiation of the epithelial lining (Qiao et al., 2022). Consequently, an inflamed gut barrier, characterized by “leaky” junctions or chronic oxidative stress, impairs the very transport mechanisms required for efficient mineral uptake. While the individual importance of these nutrients for gut health is well-documented, research on the synergistic impact of consuming multi-nutrient biofortified food products remains sparse. Future research need to address the influence of biofortified food composition and processing on cellular resilience and barrier integrity under gastrointestinal digestion, advancing from studies of single-nutrient enrichment toward the complex, interactive dynamics characteristic of multi-nutrient enriched diets.

The present study investigates the nutritional and functional effects of breads prepared from wheat grains biofortified with iodine, Se, and Zn, both individually and in combination, using two distinct matrices: non-fermented whole-wheat flatbread and fermented white sourdough. Standardized *in vitro* gastrointestinal digestion models combined with Caco-2 intestinal epithelial cell models were employed to evaluate mineral release, barrier integrity, and epithelial viability in relation to micronutrient composition and food matrix design.

2. Materials and methods

2.1. Experimental design

Field trials were conducted in 2021 at the Transitional Zone Agricultural Research Institute in Eskişehir, Turkey as described by Zou et al. (2019). To obtain wheat grains differentially enriched with Zn, I, and Se, a foliar spray program was implemented at stem elongation, heading, and early milk stages on two bread wheat (*Triticum aestivum* L.) cultivars: Bezostaja-1 and Nacibey. The treatments included KIO_3 (0.04% *w/v*), Na_2SeO_4 (0.001% w/v), and $ZnSO_4{\cdot}7H_2O$ (0.50% w/v), applied either individually or in combination. Untreated plots served as controls. After harvest, grains were processed into two flour types. Whole wheat flour was produced using a FOSS Analytical mill (Hilleröd, Denmark), while refined white flour was obtained using a Bühler MLU-202 laboratory mill (Bühler Group, Uzwil, Switzerland) after tempering to 15.5% moisture (AACC, 2009). The technological properties of these biofortified flours, relevant to dough development and bread-making performance, were characterized in our previous study (Belarbi et al., 2026).

In the present study, whole-wheat flour was used to prepare non-fermented flatbread, and white flour was used for fermented sourdough bread. Mineral concentrations in the baking ingredients are reported in Table A.1 and A.2. Baking was conducted in triplicate per treatment, with each replicate subjected to *in vitro* gastrointestinal digestion and subsequent cellular assays. Transepithelial electrical resistance (TEER) and Lucifer Yellow (LY) permeability were measured using two independent digestions per bread type, with five replicate measurements per digest. An untreated digestion control ($n = 20$), containing only the intestinal digestion solution, was used to assess matrix-related effects. Non-enriched bread controls were also included to separate matrix effects from those due to micronutrient biofortification.

Using the same intestinal digestion extracts, sulforhodamine B (SRB) and mitochondrial activity assays were performed in triplicate in 96-well plates with blanks. All TEER, LY, SRB, and mitochondrial activity assays in Caco-2 cells were conducted using digesta of individually

biofortified breads. This approach enabled controlled assessment of nutrient-specific effects on intestinal barrier function and cellular metabolism while minimizing confounding interactions present in multi-nutrient digested matrices. The experimental design was not intended as a fully factorial model; instead, analyses were conducted within each cultivar, focusing independently on biofortification treatments and bread type effects.

2.2. Bread making procedure

Bread is consumed across cultures in diverse forms, from simple unleavened flatbreads to complex fermented sourdoughs. Flatbreads, such as chapati, roti, and tortilla, are among the oldest and most widespread, valued for their simplicity and adaptability (Pasqualone et al., 2022). To replicate flatbread preparation under standardized yet realistic conditions, we adapted the protocol described by Harisha et al. (2023). Briefly, 300 g of whole wheat flour was added to 500 mL of water preheated to 85–90 °C. The dough was kneaded manually for 3 min, rested at ambient temperature (22–25 °C) for 15 min, covered to prevent surface drying, and then divided into eight equal portions. Each was shaped into a sphere, rolled to 0.5 mm thickness, and cut into 9 cm rounds. Baking was conducted on an electric hot plate for 1.5 min per side.

Sourdough bread was prepared from refined white flour, following a modified version of AACC Method No. 10-10B (AACC, 2000) to incorporate sourdough fermentation. Dough was formulated with 300 g of flour, 195 g of water, 45 g of durum wheat-based sourdough starter (Livendo levain blé dur, Bruggeman, Belgium), 0.5 g of instant dry yeast, 5.1 g of non-iodized salt (Jozo, Netherlands), 0.0075 g of ascorbic acid, and 0.21 g of malt flour. Ingredients were mixed for 8 min using a N50 Hobart mixer (Hobart, Belgium). After resting for 30 min at 23 °C in a Panem FPC 5 HR proofer (Panem, La Crèche, France), with two stretch-and-folds at 15-min intervals, the dough was divided into 150 g portions and placed in oiled aluminium pans. Fermentation went in two stages: 30 min at 24 °C and 95% relative humidity, followed by 19 h at 8 °C. Prior to baking, doughs were tempered at room temperature for 30 min. Baking was performed at 225 °C for 35 min with steam injection in a MIWE aeromat FB12 oven (Arnstein, Germany). All breads were cooled for 2 h after baking and stored at −18 °C.

2.3. In vitro gastrointestinal simulation

In vitro digestion was conducted using an adjusted INFOGEST protocol simulating human gastrointestinal conditions (Minekus et al., 2014). In the oral phase, 6 g of homogenized bread was mixed with simulated salivary fluid containing α-amylase at a 1:2 (*w*/w) ratio and incubated at 37 °C with agitation (150 rpm) for 2 min. The gastric phase was followed by adding simulated gastric fluid with pepsin to the oral bolus at a 1:1 (w/w) ratio. The pH was adjusted to 2.0 using 1 M hydrochloric acid, and the mixture was incubated for 2 h at 37 °C with constant agitation. After this step, the intestinal phase was conducted by introducing simulated intestinal fluid having pancreatin and bile salts at a 1:1 (w/w) ratio to the running digested fluid and adjusting the pH to 7.0 with sodium hydroxide, followed by an incubation for an additional 2 h. The resulting intestinal digesta was collected, yielding a total recovered volume of 48 mL. The digesta were then centrifuged at 4500 ×*g* for 15 min (Centrifuge 5804 R, Eppendorf, Germany), and filtered through 0.45 µm syringe filters (Chromafil, Machery-Nagel, Germany). The obtained supernatant was stored at −20 °C until further analysis.

Mineral intestinal release was quantified as the total mineral concentration measured in the filtered supernatants, as described in section 2.7, and corrected for procedural blanks and dilution factors. Relative bioaccessibility, hereafter referred to as bioaccessibility, was calculated as the percentage of each mineral released into filtered supernatants during digestion relative to its initial concentration in undigested bread, using an equation adapted from Roselli et al. (2020) (Eq. (1)).

$$Bioaccessibility(\%) = \left(\frac{(C_{supernatant} - C_{blank}) \times DF \times V \times 1000}{m \times C_{bread}}\right) \times 100 \quad (1)$$

where $C_{supernatant}$ is the mineral concentration in the filtered intestinal supernatant (µg/L), C_{blank} is the concentration measured in the procedural blank (µg/L), DF is the dilution factor, V is the volume of the bioaccessible fraction (L), m is the mass of the bread sample (g) and C_{bread} is the total mineral concentration in the undigested bread (µg/kg fresh weight). The factor 1000 accounts for unit conversion between g and kg, and the final multiplication by 100 expresses the result as a percentage.

2.4. Caco-2 cell culture and barrier integrity

Caco-2 cells (human colorectal adenocarcinoma line, ATCC, Molsheim, France) at passage numbers between 25 and 35 were seeded onto permeable polyethylene inserts (6.5 mm diameter, 0.4 µm pore size, 0.33 cm^2 growth area; Costar, Corning, NY, USA) placed in 24-well HTS-Transwell® plates at a density of 2×10^4 cells/cm^2. Cultures were maintained in Dulbecco's Modified Eagle Medium (Gibcon, Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (VWR, Leuven, Belgium), 1% non-essential amino acids (NEAA, Gibco, Thermo Fisher Scientific), and 1% penicillin–streptomycin (P/S, Gibco, Thermo Fisher Scientific). Differentiation was conducted for 21–23 days post-confluence at 37 °C in a humidified 5% CO_2 atmosphere (Memmert incubator, VWR International).

Transepithelial electrical resistance was then assessed using an automated REMS TEER Analyzer (World Precision Instruments, Hertfordshire, United Kingdom), and monolayers with baseline TEER values between 200 and 500 Ω·cm^2 were considered functionally intact. Paracellular permeability was assessed using LY (100 µM final concentration) as a fluorescent marker. Filtered intestinal digests, from individual biofortified breads, were diluted 1:4 in Hanks Balanced Salt Solution (HBSS) (50 µL digest, 150 µL HBSS, 2 µL LY of a 10 g/L stock solution; 200 µL total) and applied to the apical side of Caco-2 monolayers.

Lucifer yellow and TEER flux were measured after 1, 1.5, 3, and 24 h of incubation to assess time-dependent changes in barrier integrity. Lucifer yellow fluorescence in the basolateral compartment was quantified using a SpectraMax plate reader (Molecular Devices, Brussels, Belgium) at an excitation wavelength of 428 nm and emission at 530 nm. Results were expressed compared to baseline values, and increased fluorescence intensity was interpreted as indicative of impaired paracellular barrier function.

2.5. Apparent permeability

Apparent permeability (P app) was calculated using the Eq. (2) as defined by Ma et al. (2014):

$$Papp = \frac{\left(\frac{dQ}{dt}\right)}{A \times C_0} \quad (2)$$

where dQ/dt is the slope of the cumulative amount of LY transported (Q, µg) *versus* time (s), derived by linear regression over the 1–3 h time points. A is the surface area of the insert (0.33 cm^2), and C_0 is the initial apical LY concentration (100 µg/mL). Q was obtained by multiplying LY concentration in the basolateral compartment by the receiver volume (1 mL).

2.6. Mitochondrial respiration and total protein content of the Caco-2 cells

Cytotoxicity of digested conventional and biofortified bread was evaluated in differentiated Caco-2 cells seeded in 96-well plates. Cells were exposed to filtered supernatants derived from individual

biofortified breads, diluted 1:9 in HBSS, and incubated for 24 h to maximize detection of differential effects. Mitochondrial activity and total protein content, as indicators of cytotoxic responses, were assessed using the resazurin and SRB assays, respectively. For the resazurin assay, 2 µL of a 1 mg/mL stock was added to 200 µL of medium, followed by 2 h incubation at 37 °C to allow conversion of resazurin to the fluorescent resorufin. Next, the medium was transferred to a black well plate, and fluorescence was measured at an excitation and emission wavelength of 560 nm and 590 nm, respectively, using the Spectramax plate reader. The remaining cells were fixed with 10% trichloroacetic acid, incubated at 4 °C for 1 h, washed with water, air-dried, and stained with 0.4% SRB in 1% glacial acetic acid for 30 min. Next, excess dye was removed, plates were washed with glacial acetic acid, dried, and the bound stain was solubilized in 10 mM Tris base. All values were normalized to the HBSS-treated control, which did not include bread matrices.

2.7. Mineral analysis

Bread and filtered supernatants from the *in vitro* intestinal digestion were analysed for mineral concentrations. For total iodine analysis, 0.3 g of homogenized bread was weighed into 50 mL polycarbonate centrifuge tubes. Each replicate received 5 mL of ultrapure water (Milli-Q, Millipore, Belgium) and 2 mL of 25% tetramethylammonium hydroxide (TMAH). Following vortex mixing, replicates were digested in a water bath at 90 °C for 3 h. After cooling, 2 mL of a 2 µg/mL tellurium internal standard was added, and the final volume was adjusted to 50 mL with ultrapure water. Iodine concentrations were quantified using inductively coupled plasma mass spectrometry (ICP-MS; NexION 350D, PerkinElmer, USA). To assess iodine bioaccessibility, 1 mL of the filtered supernatant was combined with 1 mL of ultrapure water and 0.2 mL of TMAH, then digested under the same conditions, cooled, and diluted to 5 mL with ultrapure water. In both analyses, ERM-BB422 fish powder and ERM-BD151 skimmed milk powder were used as certified reference materials, with recoveries of 97% and 98%, respectively.

Total Se and Zn concentrations were determined *via* closed-vessel microwave-assisted acid digestion. Homogenized bread samples (0.4 g) were combined with 10 mL of 69% ultrapure nitric acid (HNO_3; Picopure) in PFA-lined vessels and digested using a Mars 6 microwave digestion system (CEM, USA). After cooling, acid digests were diluted to 50 mL with ultrapure water. Selenium concentrations were quantified by ICP-MS, while Zn concentrations were measured using inductively coupled plasma optical emission spectrometry (ICP-OES; iCAP 7200, Thermo Fisher Scientific, USA). For bioaccessibility analysis, 1 mL of the filtered supernatant was acidified with 1.5 mL of 69% HNO_3, sonicated at 50 °C for 60 min, and diluted to 10 mL prior to analysis. Isobutanol was added during Se analysis to stabilize the matrix, and yttrium was used as an internal standard for Zn to correct for instrumental variability. All measurements were performed in triplicate, and results are reported on a fresh weight basis.

2.8. Statistical analysis

Statistical differences between treatments and cultivars were evaluated using one-way or multi-way ANOVA, followed by Tukey's Honestly Significant Difference (HSD) post-hoc test ($p < 0.05$). Analyses were performed using SPSS (version 29, IBM, USA) and RStudio (version 2024.04.2, Posit PBC).

3. Results

3.1. Mineral composition of biofortified breads

Micronutrient retention in breads differed with wheat genotype, biofortification approach, and food matrix characteristics, all of which influence the nutritional quality and potential health benefits of the final product (Fig. 1). For iodine, Bezostaja-1 showed higher concentrations than Nacibey in bread under both control and biofortified conditions, mirroring results observed in the grain (data not presented). These differences likely reflect genotype-dependent iodine uptake and grain allocation during grain filling, given the limited phloem mobility of iodine (Cakmak et al., 2017). In Bezostaja-1 whole wheat flatbread, iodine concentration increased by approximately 17-fold under individual iodine application and 13-fold under multinutrient application compared to the control. In Nacibey flatbread, iodine enrichment was lower in absolute terms but still substantial, increasing by approximately 9-fold and 13-fold under individual and combined biofortification treatments, respectively. Across both cultivars biofortified white sourdough breads had lower iodine concentrations than the corresponding flatbreads. However, iodine levels were still significantly higher than in non-biofortified breads, showing 1.6- to 3.3-fold increases ($p < 0.05$).

A comparable pattern was observed for Se after biofortification. However, the response varied by genotype and food processing. In Bezostaja-1, while whole wheat flat bread showed higher enrichment efficiency (7–10-fold) than sourdough bread (6–8-fold) under both individual and combined treatments, final Se concentrations were higher in white sourdough bread. In contrast, Nacibey showed no difference in Se concentration between bread types, with enrichment averaging approximately 9-fold.

Likewise, Zn biofortification significantly enhanced Zn concentrations in both cultivars and bread types ($p < 0.05$; Fig. 1). In flatbread,

Fig. 1. Concentrations of iodine (A), selenium (B), zinc (C), and iron (D) in flatbread made from whole wheat flour and sourdough bread made from white flour. Flours were produced from wheat cultivars Bezostaja-1 and Nacibey under foliar biofortification treatments: control (no treatment); individual application of I, Se, or Zn; and combined application of I, Se, and Zn. Lowercase letters indicate significant differences among treatments within the same bread type and nutrient application; uppercase letters indicate differences between bread types within the same treatment and nutrient application ($p < 0.05$).

Table 1
Iodine (I) and Selenium (Se) intestinal release, and bioaccessibility in whole wheat flatbread and white sourdough bread. Two wheat cultivars (Bezostaja-1; Nacibey) were used across control, I-only, Se-only, and combined biofortification treatments. Intestinal release values are normalized to the initial bread mass. Lowercase letters indicate significant differences among treatments within the same bread type and nutrient application; uppercase letters indicate differences between bread types within the same treatment and nutrient application ($p < 0.05$).

Treatment	Bezostaja-1		Nacibey	
	Intestinal Release* (μg/kg)	Bioaccessibility (%)	Intestinal Release* (μg/kg)	Bioaccessibility (%)
		I		
Flat Bread				
Control	2.64 ± 0.80^{aA}	11.2 ± 3.4^{aA}	6.34 ± 1.54^{aA}	37.5 ± 7.2^{aA}
I	97.6 ± 1.7^{bA}	26.7 ± 3.8^{bA}	53.2 ± 4.2^{bA}	37.3 ± 4.2^{aA}
I, Se, Zn	68.3 ± 9.6^{cA}	24.0 ± 2.7^{bA}	50.8 ± 3.9^{bA}	43.0 ± 9.9^{aA}
Sourdough Bread				
Control	0.79 ± 0.07^{aB}	2.85 ± 1.26^{aB}	28.6 ± 1.6^{aB}	74.5 ± 8.1^{aB}
I	75.0 ± 6.5^{bB}	48.7 ± 4.8^{bB}	45.5 ± 2.1^{bB}	59.3 ± 2.0^{bB}
I, Se, Zn	48.7 ± 3.7^{cB}	45.8 ± 3.5^{bB}	35.8 ± 1.3^{cB}	48.2 ± 0.5^{bA}
		Se		
Flat Bread				
Control	15.8 ± 1.2^{aA}	83.1 ± 5.5^{aA}	18.6 ± 1.9^{aA}	82.1 ± 7.1^{aA}
Se	121 ± 37^{bA}	61.7 ± 18.2^{aA}	118 ± 12^{bA}	53.4 ± 4.6^{bA}
I, Se, Zn	92 ± 21.0^{bA}	68.0 ± 15.9^{aA}	104 ± 14^{bA}	55.4 ± 10.5^{bA}
Sourdough Bread				
Control	23.3 ± 1.9^{aB}	84.1 ± 13.6^{aA}	17.7 ± 1.2^{aA}	71.2 ± 6.7^{aA}
Se	100 ± 16^{bA}	42.4 ± 5.7^{bA}	84.2 ± 6.8^{bB}	38.1 ± 3.1^{bB}
I, Se, Zn	81.4 ± 7.6^{bA}	48.5 ± 5.4^{bA}	86.1 ± 1.2^{bA}	52.1 ± 3.7^{bA}

* Concentrations are reported as μg/kg of initial bread equivalent.

individual Zn resulted in the highest Zn accumulation, reaching 50 mg/kg in Bezostaja-1 and 23 mg/kg in Nacibey. In contrast, combined application with I and Se consistently resulted in lower zinc concentrations, decreasing to 31 mg/kg in Bezostaja-1 and 28 mg/kg in Nacibey. In sourdough breads, Zn concentrations also increased compared to the control; however, enrichment levels were lower than those observed in flatbread, and differences between individual and combined treatments were less pronounced.

Moreover, although Fe was not a target of biofortification, its concentration was influenced by Zn treatments. In whole wheat flatbread, Zn-only application increased Fe concentration relative to the control by 25% in Nacibey and 36% in Bezostaja-1, while multinutrient application resulted in lower Fe concentrations than Zn-only treatment, with reductions of 21% in Bezostaja-1 and 11% in Nacibey. Similarly, in white sourdough bread, Fe concentration decreased following multinutrient application in both cultivars, with declines of 31% in Bezostaja-1 and 45% in Nacibey compared with Zn-only treatment.

3.2. Intestinal release and bioaccessibilty

Intestinal mineral release mirrored the differences in mineral concentrations observed among the bread products; however, increases in

Table 2
Zinc (Zn) and iron (Fe) intestinal release, and bioaccessibility in whole wheat flatbread and white sourdough made from two wheat cultivars (Bezostaja-1; Nacibey) under control, Zn-only, and combined micronutrient biofortification. Statistical differences among treatments within the same bread type and nutrient application are indicated by lowercase letters ($p < 0.05$); differences between bread types within the same treatment and nutrient application are shown by uppercase letters ($p < 0.05$).

Treatment	Bezostaja-1		Nacibey	
	Intestinal Release* (mg/kg)	Bioaccessibility (%)	Intestinal Release* (mg/kg)	Bioaccessibility (%)
		Zn		
Flat Bread				
Control	0.89 ± 0.10^{aA}	6.62 ± 1.50^{aA}	1.59 ± 0.14^{aA}	11.6 ± 0.6^{aA}
Zn	1.50 ± 0.40^{abA}	2.89 ± 0.41^{aA}	1.12 ± 0.12^{bA}	4.81 ± 0.63^{bA}
I, Se, Zn	1.80 ± 0.20^{bA}	5.83 ± 0.95^{aA}	1.31 ± 0.19^{abA}	4.66 ± 0.94^{bA}
Sourdough Bread				
Control	0.93 ± 0.42^{aA}	22.6 ± 10.8^{aA}	0.87 ± 0.08^{aB}	19.1 ± 0.7^{aB}
Zn	0.76 ± 0.16^{aB}	8.53 ± 1.47^{aB}	1.89 ± 0.07^{bB}	18.8 ± 0.9^{aB}
I, Se, Zn	0.90 ± 0.14^{aB}	12.4 ± 2.1^{aB}	1.58 ± 0.02^{cA}	16.6 ± 0.2^{bB}
		Fe		
Flat Bread				
Control	1.38 ± 0.15^{aA}	5.33 ± 1.13^{aA}	1.16 ± 0.17^{aA}	5.66 ± 0.25^{aA}
Zn	2.05 ± 0.58^{aA}	5.58 ± 0.98^{aA}	2.02 ± 0.32^{bA}	7.96 ± 2.03^{aA}
I, Se, Zn	1.71 ± 0.01^{aA}	8.57 ± 0.35^{bA}	1.12 ± 0.14^{aA}	4.89 ± 0.85^{aA}
Sourdough Bread				
Control	0.44 ± 0.15^{aB}	4.12 ± 1.84^{aA}	0.39 ± 0.27^{aB}	2.86 ± 1.43^{aB}
Zn	0.45 ± 0.11^{aB}	3.09 ± 0.90^{aB}	0.54 ± 0.00^{aB}	6.00 ± 0.58^{bA}
I, Se, Zn	0.29 ± 0.08^{aB}	3.80 ± 1.02^{aB}	0.25 ± 0.08^{aB}	3.42 ± 1.11^{abA}

* Concentrations are reported as mg/kg of initial bread equivalent.

total mineral concentration were not consistently accompanied by proportional increases in relative bioaccessibility. Selenium release during intestinal digestion increased significantly following biofortification treatments, reaching 81–121 μg/kg, compared with an average of 19 μg/kg in control breads ($p < 0.05$; Table 1). Despite the higher Se release, bioaccessibility declined in biofortified breads, averaging 58% in Bezostaja-1 and 54% in Nacibey, compared with 83% and 76.5%, respectively, in the corresponding controls. Importantly, Se release and bioaccessibility were not affected by bread type or biofortification strategy ($p > 0.05$).

For iodine, differences were observed between cultivars and bread types. In Bezostaja-1, biofortification increased iodine bioaccessibility relative to the control in both bread types, rising from 11% to 25% in whole wheat flatbread and from 3% to 47% in white sourdough bread. In contrast, in the Nacibey cultivar, iodine bioaccessibility in whole wheat flatbread did not differ between control and biofortified treatments, averaging approximately 40%. In white sourdough bread, however, iodine bioaccessibility in biofortified breads was 28% lower than in the corresponding control. Nevertheless, in both cultivars, iodine bioaccessibility was consistently higher in sourdough bread than in flatbread. This indicates that, despite lower absolute iodine concentrations in sourdough bread, a larger fraction of iodine became bioaccessible during gastrointestinal digestion.

Moreover, in whole wheat flatbreads, individual Zn biofortification reduced Zn bioaccessibility by approximately 55–60% relative to the control, which averaged 6.6% in Bezostaja-1 and 11.6% in Nacibey (Table 2). Multinutrient treatment partially mitigated this reduction by increasing Zn bioaccessibility to 5.83% in Bezostaja-1 and 4.66% in Nacibey. In contrast, sourdough breads showed higher Zn release and sustained bioaccessibility across treatments. In Nacibey, Zn bioaccessibility reached approximately 19% under Zn-only treatment and remained similar under multinutrient biofortification, whereas in Bezostaja-1 it increased from 8.5% to 12.4% following multinutrient treatment. These patterns indicate that, unlike flatbread, sourdough processing moderated the decline in Zn bioaccessibility associated with high Zn concentrations.

In addition, it was observed that Zn biofortification increased Fe bioaccessibility by 109% in the sourdough formulations of the Nacibey cultivar and by 61% in the multi-nutrient biofortified flatbread of Bezostaja-1.

3.3. Intestinal cell viability after biofortified bread digestion

The maintenance of epithelial cell viability and structural integrity is essential for maintaining gut function and health. Exposure to digesta derived from control flatbread and sourdough breads resulted in a significant reduction in both mitochondrial activity and protein content compared to the untreated (HBSS) cells (Fig. 2, Table A.3). Specifically, resazurin values declined from 10,985 in the untreated control to 7955 and 8881 in flatbread and sourdough digesta, respectively ($p < 0.05$). A corresponding decline in SRB values was also observed (3.95 for flatbread and 4.60 for sourdough *vs.* 9.14 for HBSS ($p < 0.05$). These results show that the digestive matrix in general decreases epithelial viability and density, irrespective of fermentation method.

However, Se biofortification significantly enhanced mitochondrial activity in both bread matrices. Flatbread biofortified with Se yielded and resazurin value of 9704, while Se-enriched sourdough bread reached 10,280, the latter significantly exceeding its non-biofortified control ($p < 0.05$) and closely approximating the untreated cells. The SRB value for Se-enriched sourdough bread was 6.86, close to the HBSS control, indicating partial preservation of cell mass. For Zn-biofortified bread, the effects were matrix-dependent, with mitochondrial activity higher in sourdough at 9937 compared with 6484 in flatbread ($p < 0.05$). SRB values, however, remained low, showing that the impact did not extend beyond mitochondrial function. In contrast, iodine biofortification did not show any effects in either assay, with resazurin values for enriched breads remaining statistically similar to their controls.

3.4. Intestinal barrier integrity and paracellular permeability dynamics in response to biofortified bread

Differentiated intestinal epithelial cells exhibit higher resilience to cytotoxic stress than undifferentiated cells; therefore, a ¼ dilution of the digests was selected to capture early barrier responses without inducing excessive damage. This concentration enabled evaluation of subtoxic effects at earlier time points, expanding the temporal resolution beyond the conventional 24-h endpoint. The results in absolute values are provided in Table A.4, and the corresponding percentages relative to the untreated cells (HBSS baseline) are shown in the Figs. 3 and 4.

The baseline TEER in the HBSS medium was 1817 Ω/cm^2, confirming intact monolayer integrity prior to treatment. After 30 min of exposure to non-biofortified bread digesta, TEER values remained statistically unchanged across all groups: HBSS (1909 Ω/cm^2), flatbread (1909 Ω/cm^2), and sourdough (1590 Ω/cm^2) ($p > 0.05$). However, LY flux indicated that short-term exposure of the sourdough digesta group showed a significant 10% reduction in LY permeability relative to untreated cells ($p < 0.01$), suggesting a transient tightening of the

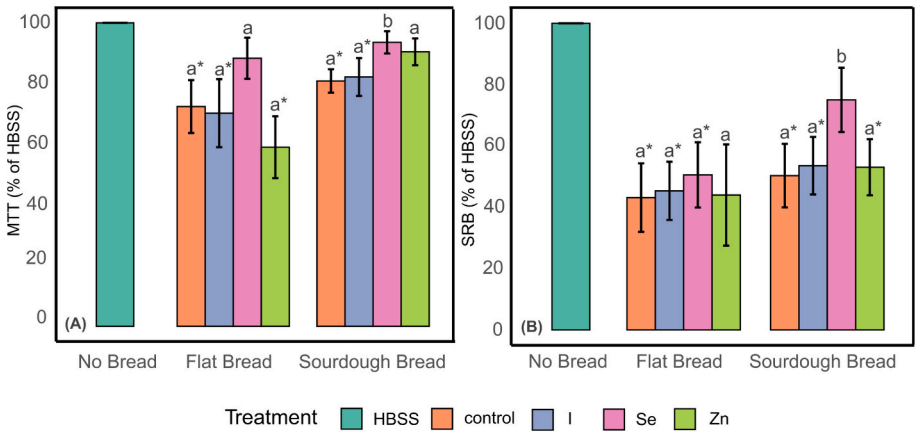

Fig. 2. Percentage of mitochondrial activity (resazurin assay (A)) and total cellular protein content (SRB assay (B)) compared to the untreated cells in HBSS medium (mean ± SD, $n = 18$). Samples included flatbread and sourdough bread, made from whole wheat flour and white flour, respectively, comprising untreated (control) and singly biofortified variants (I, Se, Zn) made from Bezostaja-1 wheat cultivar. HBSS results were used as the reference for maximum cell viability. Asterisks denote statistically significant differences compared to the HBSS ($p < 0.05$). Different letters indicate significant differences between biofortified samples and their respective controls within each bread type (flatbread and sourdough bread) ($p < 0.05$).

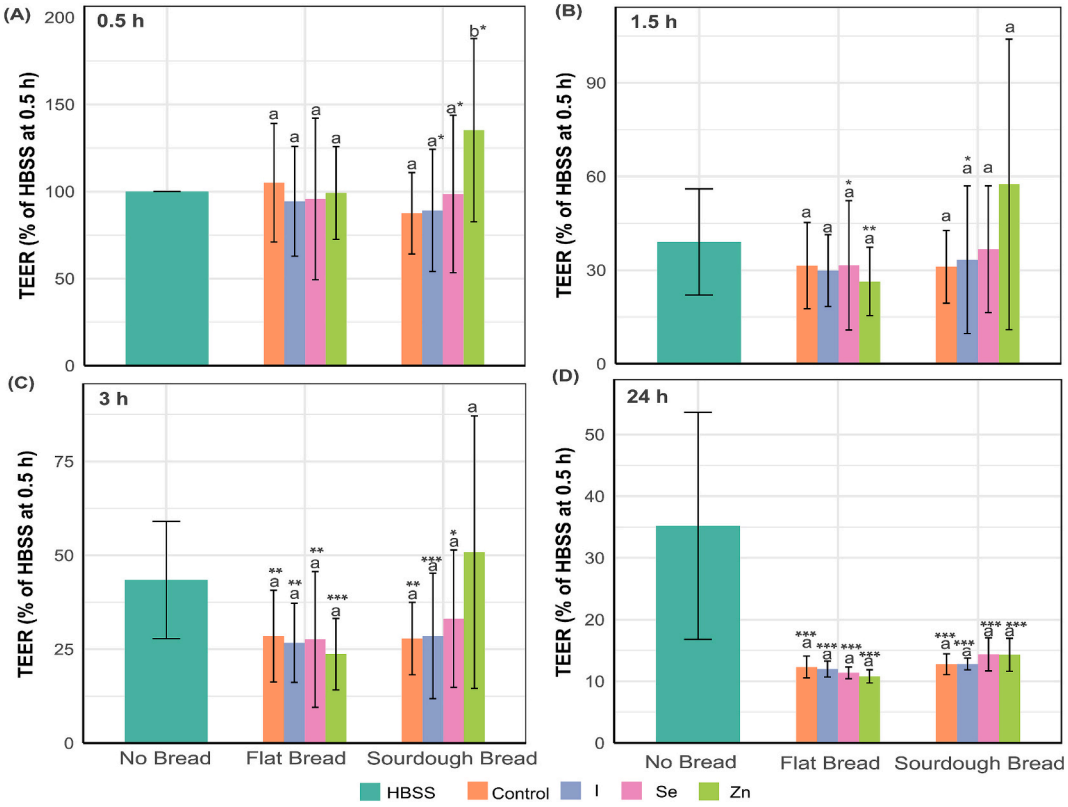

Fig. 3. Percentage of trans-epithelial electrical resistance (TEER) compared to the HBSS medium at the starting phase (no bread $n = 20$, bread $n = 10$). Measurements were taken at different time points: 0.5 h (A), 1.5 h (B), 3 h (C), 24 h (D). Intestinal digesta included flatbread and sourdough bread, made from whole wheat flour and white flour, respectively, comprising untreated (control) and singly biofortified variants (I, Se, Zn) made from Bezostaja-1 wheat cultivar. Untreated (HBSS) cells were used as the reference for maximum barrier integrity and tight junction function. Asterisks denote statistically significant differences compared to the HBSS at initial measurement ($p < 0.05$). Different letters indicate significant differences between biofortified samples and their respective controls within each bread type (flatbread and sourdough bread) ($p < 0.05$).

epithelial barrier in response to physicochemical stress brought by sourdough digest components (Fig. 3). At 1.5 h, a time point reflecting early epithelial stress responses, TEER had declined to a mean of 568 Ω/cm^2 across all treatments, and LY flux increased by about 4.8-fold. These shifts suggest early signs of barrier compromise, but no significant differences were observed between HBSS, flatbread, and sourdough groups ($p > 0.05$), indicating a general loss of resistance not specific to treatment.

By 3 h, a mid-stage response window, LY rates remained comparable among groups, while TEER in the flatbread and sourdough conditions showed an 11% and 8% decrease, respectively, relative to HBSS ($p < 0.01$). This suggests a modest but measurable loss of epithelial integrity associated with digesta exposure. At 24 h, corresponding to late-stage effects and potential cumulative cellular damage, TEER across all conditions had fallen below the epithelial integrity threshold of 300 Ω/cm^2, while LY permeability exceeded 200-fold relative to baseline in most treatments. These results indicate substantial epithelial barrier damage by 24 h, with control flatbread and sourdough bread digesta exerting comparable long-term effects on monolayer integrity and paracellular permeability.

When the bread matrices were further stratified by micronutrient biofortification, distinct patterns emerged, particularly in early-phase responses. Digesta of biofortified sourdough breads enriched with iodine, Se, and Zn showed significantly higher TEER and lower LY flux after 30 min compared to the untreated cells, indicating a protective barrier effect. Iodine-enriched breads behaved similarly to their non-biofortified counterparts beyond the initial exposure period. However, Se-enriched flatbread exhibited higher TEER at 1.5 h, and Se-enriched sourdough showed significantly decreased LY flux at the same time point, suggesting a transient protective response. However, this effect was not sustained at 3 or 24 h (Fig. 4).

Zinc biofortified bread produced the most pronounced matrix-specific effects. When delivered *via* sourdough, initial TEER was approximately 15% higher than in the non-biofortified sourdough and remained significantly elevated through 24 h, before falling in line with the other breads. Parallel measurements showed that LY flux in Zn-sourdough digesta was delayed at both 1.5 h ($p < 0.05$) and 24 h ($p < 0.01$), with 30% lower permeability compared to its flatbread counterpart (Fig. 4). In contrast, Zn delivered *via* flatbread conferred no early benefit and by 24 h was associated with a 20% decrease in TEER and a 35% increase in LY flux relative to its own non-biofortified control ($p < 0.05$, Table A.4).

Moreover, the apparent permeability coefficient of LY was significantly affected by treatment, bread type, and their interaction ($p < 0.001$, Table 3). Across bread types, control flat bread exhibited the highest permeability (4.01×10^{-6} cm/s), whereas Zn-biofortified sourdough showed the lowest (2.09×10^{-6} cm/s). In flatbread, iodine and Se, and in sourdough, Zn, were associated with reduced Papp values, whereas iodine in sourdough was associated with increased Papp values.

4. Discussion

The incorporation of agronomically biofortified wheat flour effectively increased the concentrations of I, Se, and Zn within the bread matrix. However, the magnitude of micronutrient enhancement was not uniform across bread formulations, indicating that the efficacy of biofortification depends not only on the intrinsic nutritional quality of the

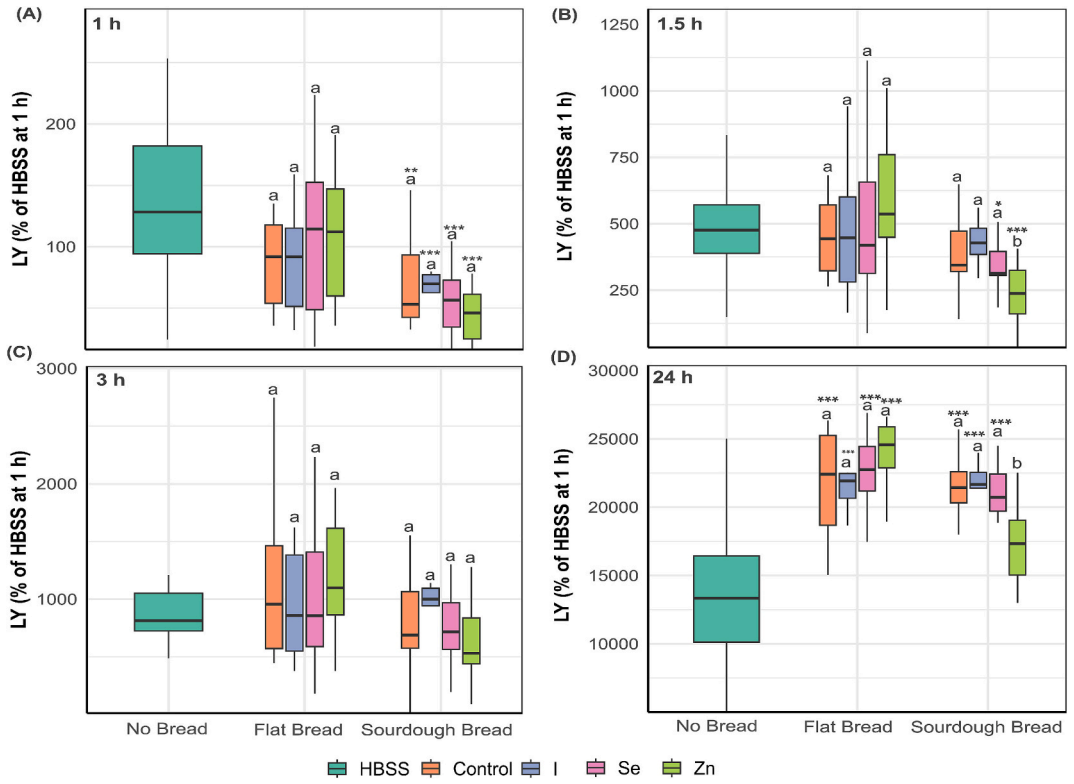

Fig. 4. Percentage of lucifer yellow (LY) compared to the HBSS medium at the starting phase (no bread n = 20, bread n = 10). Measurements were taken at different time points: 1 h (A), 1.5 h (B), 3 h (C), 24 h (D). Intestinal digesta included flatbread and sourdough bread, made from whole wheat flour and white flour, respectively, comprising untreated (control) and singly biofortified variants (I, Se, Zn) made from Bezostaja-1 wheat cultivar. Untreated (HBSS) cells were used as the reference for maximum barrier integrity and tight junction function. Asterisks denote statistically significant differences compared to the HBSS at initial measurement ($p < 0.05$). Different letters indicate significant differences between biofortified samples and their respective controls within each bread type (flatbread and sourdough bread) ($p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
Apparent permeability coefficient (Papp) of lucifer yellow in Caco-2 monolayers exposed to different bread types and treatments. Different superscript letters within the same bread type indicate significant differences between Control, I, Se, Zn (Tukey's test, $p < 0.05$). An asterisk (*) indicates significant difference from HBSS of the same bread type ($p < 0.05$).

Treatment	Papp (10^{-6} cm/s)	
	Flat Bread	Sourdough Bread
Control	$4.01 \pm 0.32^{a*}$	$2.47 \pm 0.09^{a*}$
I	$3.13 \pm 0.39^{b*}$	$3.21 \pm 0.31^{b*}$
Se	$3.54 \pm 1.08^{c*}$	2.58 ± 0.17^{a}
Zn	$3.83 \pm 0.23^{a*}$	2.09 ± 0.45^{c}
	No Bread	
HBSS**	2.38 ± 0.00	

** Hanks Balanced Salt Solution (HBSS).

raw material but also on processing and formulation factors (Fig. 1).

Biofortified whole wheat flatbreads contained higher levels of I, Zn, and Fe than biofortified white sourdough breads across both cultivars. This discrepancy is largely attributable to the flour extraction rate. While whole wheat flour retains the mineral-dense bran and germ fractions, refined white flour is composed almost exclusively of the starchy endosperm, which possesses inherently lower micronutrient reserves (Miller & Welch, 2013). Fermentation and thermal processing may also have contributed to the observed variation. Flatbreads were produced without fermentation and baked under high-temperature, short-duration conditions, whereas sourdough breads underwent extended fermentation and baking. These distinct baking profiles likely influenced the food matrix and mineral distribution. In particular, sourdough fermentation can reduce phytate levels, potentially increasing mineral interactions and solubility. In addition, Maillard reactions during baking may generate melanoidins with metal-binding properties, which could influence mineral retention. Nevertheless, these processing-related effects do not appear to compensate for the lower initial concentrations of I, Zn, and Fe associated with the use of refined white flour in sourdough bread preparation.

Selenium showed a divergent response. In Bezostaja-1, Se concentrations were higher in sourdough bread than in flatbread across both individual and combined biofortification treatments, while in Nacibey, Se levels did not differ between bread types. This variation likely reflects differences in Se transformation during fermentation and baking. Microbial metabolism during sourdough fermentation facilitates the bioconversion of inorganic selenium species (selenate and selenite) into organic analogues, specifically selenomethionine (SeMet) and selenocysteine (SeCys). These organic forms exhibit enhanced stability within the food matrix during thermal processing compared to their inorganic precursors, thereby improving Se retention in the final crumb (Di Nunzio et al., 2018). The divergent results between cultivars indicate that wheat genotype influences the initial Se speciation profile within the grain, thereby dictating its behaviour during fermentation and baking (Cubadda et al., 2010).

However, these increases did not consistently translate into higher bioaccessibility. The effectiveness of nutrient delivery was shaped not only by the biofortification approach, but also by intrinsic features of the food matrix. Despite the increase in Se concentration and intestinal release, the relative bioaccessibility of Se decreased under biofortified conditions (Table 1). This was evident in Bezostaja-1, where bioaccessibility declined from approximately 83% in control sourdough breads to 42% and 48.5% following biofortification. A similar trend was

observed for Nacibey in both sourdough and flatbread. This inverse relationship between Se concentration and its fractional bioaccessibility aligns with the saturation effects reported by Sánchez-Martínez et al. (2015). Their data suggest that excessive Se loadings may promote stronger interactions with dietary fibres or matrix proteins, whereby Se becomes less bioaccessible.

For iodine, our findings for Bezostaja-1 and Nacibey show that flour type and the food matrix influence iodine bioaccessibility (Table 1). This aligns with Cakmak et al. (2020), who reported that white-flour bread, produced as standard yeast-leavened loaves, had higher iodine bioaccessibility (82–92%) than whole-wheat bread (50–76%). Although baking did not reduce total iodine levels, the lower bioaccessibility observed in whole-wheat bread was attributed to matrix effects associated with the presence of bran and fibre. Moreover, their study demonstrated that co-biofortification of iodine with Zn and Se did not compromise iodine bioaccessibility, which we also observed in our study in both digested Bezostaja-1 sourdough and flatbread. However, an exception was observed in Nacibey sourdough, where Zn bioaccessibility was significantly lower in the co-biofortified bread than in the single-nutrient treatment. In non-cereal systems, Zhou et al. (2024) reported a broad range in the bioaccessibility of iodine from seaweed (18.5–89.0%), with values showing a negative correlation with total fibre content ($r = -0.28$, $p < 0.05$) and a positive correlation with the proportion of inorganic iodine ($r = 0.50$, $p < 0.01$). These interactions were attributed to strong iodine–protein interactions during digestion, which restricted iodine solubilization even in samples with high total iodine concentration.

Zinc appeared as the most matrix-dependent mineral, with both its intestinal release and bioaccessibility influenced by the combined effects of flour composition, fermentation, and biofortification treatment ($p < 0.05$, Table 2). For example, for the Bezostaja-1 cultivar, whole wheat co-biofortified flatbreads produced without fermentation increased Zn concentrations released from the bread in the intestinal phase to 1.80 mg/kg, compared to 0.89 mg/kg in the control bread. Yet the mineral's bioaccessibility remained critically low, falling to from 6.62% in the control to 5.83% and 2.89% in multi-nutrient and individually biofortified flatbreads, respectively.

This pattern reflects the inhibitory role of phytic acid (PA) within the bread digesta during the intestinal phase. Phytic acid remains prevalent in whole-grain formulations and strongly chelates divalent cations, such as Zn^{2+} and Fe^{2+}, forming insoluble complexes that resist enzymatic degradation and limit mineral solubilization (Brouns, 2021; Miller et al., 2007). These observations are supported by the [PA]:[Zn] molar ratios of the starting flours (data not presented). In the case of whole-wheat flour, the ratio was 37 in the control and between 22 and 24 in the biofortified flour, both well above the critical threshold of 15 known to impair Zn absorption in both *in vitro* and human studies (Gibson et al., 2006). Conversely, the biofortified white flour exhibited a lower ratio (12−13), compared to 31 in its respective control. Moreover, the lower Zn release observed in the intestinal digesta (~pH 7.0) compared to the gastric phase (~pH 2.0) (data not shown) confirms that these phytate–Zn complexes, dictated by the initial flour composition, precipitate as acidity decreases. Unlike these divalent cations, I and Se are typically present in anionic (I^-) or organic (*e.g.*, SeMet) forms, which do not form insoluble precipitates with phytate due to their distinct chemical speciation.

Reducing phytate levels, however, improves Zn bioaccessibility. For example, in sourdough bread produced with multi-nutrient biofortified white flour, bioaccessibility reached 12% in Bezostaja-1 and 17% in Nacibey, compared with 6% and 5%, respectively, in whole wheat flatbread. This improvement is linked to use of refined flour and fermentation-driven phytate hydrolysis. White flour, obtained through milling, contains lower amounts of phytate due to the removal of bran and germ fractions, thereby reducing the pool of endogenous chelating agents that limit mineral solubility. In parallel, sourdough fermentation creates an acidic environment that activates endogenous cereal and microbial phytases, promoting the progressive degradation of phytate into lower inositol phosphates with reduced mineral-binding capacity (Sandberg et al., 1999). Consistent with this mechanism, *Lactobacillus*-derived phytases have been shown to degrade 60–90% of phytate within 12–24 h of fermentation, indicating that the 19-h fermentation period applied in the present study likely achieved comparable reductions (Rizzello et al., 2010). Similar reductions have also been reported in cereal-based systems (Karaduman et al., 2024). The combined effect of lower initial phytate content and enzymatic hydrolysis enhances Zn solubility during digestion and facilitates its release from the food matrix. In addition, Zn biofortification may contribute by partially occupying residual phytate binding sites, reducing competition with other divalent cations and indirectly supporting mineral availability (Hurrell & Egli, 2010).

Baking conditions further influence these interactions through their effect on matrix structure. The longer baking time used for sourdough bread promotes starch gelatinization and protein denaturation, resulting in a more open and porous structure that increases the surface area accessible to digestive enzymes. This structure facilitates matrix disintegration during digestion and supports mineral release. In contrast, the short, high-temperature baking typical of flatbreads produces a denser structure that can limit access of digestive enzymes and reduce mineral accessibility.

Beyond mineral release and bioaccessibilty, the physiological relevance of biofortified breads extends to their effects on intestinal epithelial function. Nutrient bioaccessibility alone does not guarantee nutritional benefit if the epithelial barrier is compromised or unable to absorb nutrients efficiently. Our results shows that both bread matrix composition and micronutrient biofortification influence epithelial responses under the same simulated digestive conditions (Fig. 3, Fig. 4). Sourdough fermentation provided a transient benefit to tight junction regulation. Within 30 min of exposure, LY flux decreased by approximately 10% compared to the HBSS control ($p < 0.01$), indicating a mild tightening of the paracellular barrier. This observation is consistent with established mechanisms by which fermentation-derived compounds such lactic acid, short-chain fatty acids, and bioactive peptides, modulate epithelial barrier integrity. Experimental studies have demonstrated that such compounds, primarily derived from fermented dairy products (*e.g.*, yogurt, kefir) and fermented vegetable and cereal-based foods (*e.g.*, kimchi, sourdough bread), can enhance tight junction function by upregulating the expression of key proteins such as claudin-1 and occludin, leading to reduced paracellular permeability (Roselli et al., 2006; Ulluwishewa et al., 2011). These effects are thought to be mediated, at least in part, through activation of intracellular signalling cascades involving mitogen-activated protein kinases and protein kinase C, which regulate both tight junction assembly and cytoskeletal dynamics. The transient improvement in barrier function observed here suggests that sourdough fermentation products may trigger early-phase signalling responses that stabilize tight junctions and restrict solute flux across the epithelial layer.

Moreover, Zn biofortification delivered through sourdough matrices provided more sustained barrier support, as reflected by higher TEER values and delayed paracellular flux both at the onset and 24 h after treatment, compared with flatbread ($p < 0.05$). TEER values in Zn-sourdough treatments remained approximately 15% higher than in non-biofortified sourdough, while LY permeability was reduced by up to 30% (Table A.4, $p < 0.01$). These findings are consistent with the known role of Zn in maintaining epithelial homeostasis through structural and signalling mechanisms. In an *in vitro* study using Caco-2 cell monolayers, Wang et al. (2013) demonstrated that Zn supplementation increased TEER and upregulated the expression of tight junction proteins including occludin and tricellulin, both key regulators of paracellular permeability. Similarly, Zhang and Guo (2009), in a weaned piglet model, reported that dietary Zn improved intestinal barrier function by increasing the expression of occludin and zonula occludens-1, which directly strengthened tight junctions and reduced intestinal leakage.

Beyond structural support, Zn also exerts potent anti-inflammatory and antioxidant effects that further stabilize the epithelial barrier. Finamore et al. (2008) showed that Zn deficiency in Caco-2 cells led to tight junction disassembly, elevated oxidative stress, and increased neutrophil transmigration, processes that compromise epithelial integrity. By contrast, Zn sufficiency preserved both tight junction architecture and redox balance, outcomes that parallel the improved TEER and reduced permeability observed in the Zn sourdough breads in the current study.

However, this beneficial effect proved to be food matrix dependent. In breads made with wholegrain flatbread, Zn biofortification conferred no significant improvement in barrier function. Instead, TEER declined by approximately 20% and LY flux increased by 35% at 24 h compared to the non-fortified flatbread control. This disparity stems from higher phytate and fibre content in the wholegrain matrix. In contrast, sourdough fermentation, mediated through the enzymatic degradation of phytate and modification of the mineral-binding environment, appears to mitigate this inhibitory effect, allowing Zn to exert its full biological potential in maintaining epithelial barrier integrity.

Selenium enrichment was similarly effective in enhancing epithelial cell metabolic activity and protein content; though, its effects were limited to the early phase of exposure and did not extend to measurable improvements in barrier integrity (Fig. 2). In both flatbread and sourdough bread matrices, Se-enriched breads increased mitochondrial dehydrogenase activity, with mitochondrial activity values reaching 10,280 in Se-sourdough, compared to 8881 in the non-biofortified counterpart (Table A.3). Similarly, total cellular protein (SRB assay) increased from 4.60 in the control bread to 6.86 following Se enrichment. These outcomes are consistent with Se's established role in supporting cellular antioxidant defences, primarily through its incorporation into glutathione peroxidase, which reduces oxidative stress and protects cellular components from damage. Previous research in intestinal epithelial models and animal systems has demonstrated that Se supplementation can attenuate oxidative injury, preserve cell viability, and modulate key stress-responsive pathways, including the nuclear factor kappa-light-chain-enhancer of activated B cells and the mitogen-activated protein kinase cascade (He et al., 2025; Qiao et al., 2022). The absence of significant effects on transepithelial resistance or paracellular permeability in the present study indicated that Se's influence was limited to early cytoprotective effects without sustained reinforcement of epithelial barrier structure.

In contrast, I-biofortified bread digesta produced no significant effects on any of the parameters assessed, including epithelial viability, protein content, transepithelial electrical resistance, or paracellular permeability. This absence of response is consistent with iodine's primary physiological role in thyroid hormone synthesis and systemic metabolic regulation, with no established function in modulating intestinal epithelial integrity or acute stress responses (Gong et al., 2024).

These findings indicate that enrichment with individual micronutrients can influence intestinal barrier integrity and cellular metabolic activity under physiologically relevant conditions. However, *in vitro* digestion models and Caco-2 monolayers but do not fully replicate the physiological complexity of the human gastrointestinal tract, including mucus barriers, microbiota interactions, and dynamic luminal conditions. In addition, the exclusive focus on the Bezostaja-1 and Nacibey cultivars limits generalizability due to genotype-dependent differences in phytate content and matrix composition affecting mineral release and transport. Moreover, the absence of stable isotope labelling further limits the ability to precisely track the fate of the studied micronutrients. Future studies may incorporate speciation analysis, co-culture systems, intestinal organoids, or human intervention trials to better evaluate absorption and systemic effects. Furthermore, investigating multi-micronutrient interactions within digesta, alongside comparisons with isolated micronutrients, will help clarify nutrient–nutrient and matrix–nutrient interactions in complex dietary systems.

5. Conclusion

While individual and multinutrient biofortification increases the concentrations of I, Se, and Zn in wheat, the nutritional value of these minerals is largely shaped by the food matrix. Whole wheat flatbreads tend to retain higher I and Zn concentrations due to the mineral-rich bran and germ fractions, whereas Se retention appears more dependent on the wheat variety and the use of sourdough fermentation. However, during intestinal digestion, both I and Zn exhibited higher relative bioaccessibility rates in white sourdough breads than in whole wheat flatbreads, indicating that flour refinement and fermentation collectively enhanced mineral availability. On the other hand, Se enrichment did not consistently translate into a higher relative bioaccessibility, due to the effect of saturation, *i.e.* the storage of the enriched Se in less bioaccessible forms and fractions of the grain. At the level of the gastrointestinal cells, Se-enriched breads increased metabolic activity, while Zn-enriched sourdough breads contributed to epithelial barrier stability by strengthening tight junctions. These observations indicate that effective biofortification involves more than just increasing mineral levels; it requires a thoughtful approach to how agronomic choices and food processing work together to ensure that nutrients are available and functional for the consumer.

CRediT authorship contribution statement

Hind Belarbi: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Fassil Kebede:** Writing – review & editing, Conceptualization. **Fleur Lambrecht:** Investigation. **Hanne Lampaert:** Investigation. **Charlotte Grootaert:** Writing – review & editing, Resources, Methodology, Data curation. **Ingrid De Leyn:** Writing – review & editing, Resources, Methodology. **Filip Van Bockstaele:** Writing – review & editing, Methodology. **Tom Van de Wiele:** Resources, Methodology. **Ismail Cakmak:** Writing – review & editing, Supervision, Resources. **Gijs Du Laing:** Writing – review & editing, Supervision, Conceptualization.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this study, the author(s) used ChatGPT to improve the readability and language of the manuscript. After utilizing this tool, the author(s) reviewed and edited the content as needed and take full responsibility for the final published article.

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Declaration of competing interest

The authors 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. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2026.103882.

Data availability

Data will be made available on request.

References

AACC. (2000). Method 10-10B: Basic Straight Dough Bread-Baking Method. In *Approved Methods of Analysis* (10th ed.). *American Association of Cereal Chemists* (AACC).

AACC. (2009). Method 26-10.02: Experimental milling, determination of flour extraction. In *Approved methods of analysis* (11th ed.). American Association of Cereal Chemists (AACC).

Ashraf, S. A. (2025). Food fortification as a sustainable global strategy to mitigate micronutrient deficiencies and improve public health. *Discover Food, 5*(1), 1–24. https://doi.org/10.1007/S44187-025-00512-5/TABLES/3

Belarbi, H., Kebede, F., De Leyn, I., Van Bockstaele, F., Vermeir, P., Savaşlı, E., Cakmak, I., & Du Laing, G. (2026). Effects of iodine, selenium, and zinc biofortification on wheat flour and dough properties for bread making. *LWT*, 119099. https://doi.org/10.1016/J.LWT.2026.119099.

Bouis, H. E., & Saltzman, A. (2017). Improving nutrition through biofortification: A review of evidence from HarvestPlus, 2003 through 2016. Global. *Food Security, 12*, 49–58. https://doi.org/10.1016/J.GFS.2017.01.009

Brouns, F. (2021). Phytic acid and whole grains for health controversy. *Nutrients, 14*(1), 25. https://doi.org/10.3390/NU14010025

Cakmak, I., Marzorati, M., Van Den Abbeele, P., Hora, K., Holwerda, H. T., Yazici, M. A., … Du Laing, G. (2020). Fate and bioaccessibility of iodine in food prepared from agronomically biofortified wheat and rice and impact of cofertilization with zinc and selenium. *Journal of Agricultural and Food Chemistry, 68*(6), 1525–1535. https://doi.org/10.1021/acs.jafc.9b05912

Cakmak, I., Prom-u-thai, C., G Guilherme, L. R., Rashid, A., Hora, K. H., Yazici, A., Savasli, E., Kalayci, M., Tutus, Y., Phuphong, P., Rizwan, M., D Martins, F. A., Dinali, G. S., Ozturk, L., & John White Cakmak, P. I. (2017). Iodine biofortification of wheat, rice and maize through fertilizer strategy. Plant and Soil, 418, 319–335. https://doi.org/10.1007/s11104-017-3295-9.

Cubadda, F., Aureli, F., Ciardullo, S., D'Amato, M., Raggi, A., Acharya, R., … Prakash, N. T. (2010). Changes in selenium speciation associated with increasing tissue concentrations of selenium in wheat grain. *Journal of Agricultural and Food Chemistry, 58*(4), 2295–2301. https://doi.org/10.1021/JF903004A

Di Nunzio, M., Bordoni, A., Aureli, F., Cubadda, F., & Gianotti, A. (2018). Sourdough fermentation favorably influences selenium biotransformation and the biological effects of flatbread. Nutrients 2018, vol. 10, page 1898, 10(12), 1898. Doi;https://doi.org/10.3390/NU10121898.

Finamore, A., Massimi, M., Devirgiliis, L. C., & Mengheri, E. (2008). Zinc deficiency induces membrane barrier damage and increases neutrophil transmigration in caco-2 cells. *Journal of Nutrition, 138*(9), 1664–1670. https://doi.org/10.1093/JN/138.9.1664

Gapp, K., Steenwyk, G., Germain, P. L., Matsushima, W., M Rudolph, K. L., Manuella, F., Roszkowski, M., Vernaz, G., Ghosh, T., Pelczar, P., Mansuy, I. M., & Miska, E. A. (2024). Current iodine status in Europe and Türkiye in the light of the World Health Organization European region 2024 report: Are we losing our achievements? *Endocrinol res Pract.* https://doi.org/10.5152/erp.2025.24594.

Gibson, R. S., Perlas, L., & Hotz, C. (2006). Improving the bioavailability of nutrients in plant foods at the household level. *The Proceedings of the Nutrition Society, 65*(2), 160–168. https://doi.org/10.1079/PNS2006489

Gong, B., Meng, F., Wang, X., Han, Y., Yang, W., Wang, C., & Shan, Z. (2024). Effects of iodine intake on gut microbiota and gut metabolites in Hashimoto thyroiditis-diseased humans and mice. *Communications Biology, 7*(1), 136. https://doi.org/10.1038/S42003-024-05813-6

Gupta, S., Brazier, A. K. M., & Lowe, N. M. (2020). Zinc deficiency in low- and middle-income countries: Prevalence and approaches for mitigation. *Journal of Human Nutrition and Dietetics, 33*(5), 624–643. https://doi.org/10.1111/JHN.12791

Harisha, R., Singh, S. K., Ahlawat, A. K., Narwal, S., Jaiswal, J. P., Singh, J. B., … Mahendru-Singh, A. (2023). Elucidating the effects on polyphenol oxidase activity and allelic variation of polyphenol oxidase genes on dough and whole wheat-derived product color parameters. *International Journal of Food Properties, 26*(2), 2716–2731. https://doi.org/10.1080/10942912.2023.2252196

He, L., Zhang, L., Peng, Y., & He, Z. (2025). Selenium in cancer management: Exploring the therapeutic potential. Frontiers. *Oncology, 14*, Article 1490740. https://doi.org/10.3389/FONC.2024.1490740

Hurrell, R., & Egli, I. (2010). Iron bioavailability and dietary reference values. *The American Journal of Clinical Nutrition, 91*(5), 1461S–1467S. https://doi.org/10.3945/AJCN.2010.28674F

Karaduman, Y., Gülbandılar, A., Akın, A., Doğan, S., & Savaşlı, E. (2024). Zinc and selenium biofortification of sourdough breads with agronomically biofortified whole wheat flour. *Journal of Cereal Science, 118*, Article 103952. https://doi.org/10.1016/J.JCS.2024.103952

Lončarić, Z., Ivezić, V., Kerovec, D., & Rebekić, A. (2021). Foliar zinc-selenium and nitrogen fertilization affects content of zn, fe, se, p, and cd in wheat grain. *Plants, 10*(8). https://doi.org/10.3390/plants10081549

Miller, B. D. D., & Welch, R. M. (2013). Food system strategies for preventing micronutrient malnutrition. *Food Policy, 42*, 115–128. https://doi.org/10.1016/j.foodpol.2013.06.008

Miller, L. V., Krebs, N. F., & Hambidge, K. M. (2007). A mathematical model of zinc absorption in humans as a function of dietary zinc and phytate,2. *The Journal of Nutrition, 137*(1), 135–141. https://doi.org/10.1093/JN/137.1.135

Ning, P., Fei, P., Wu, T., Li, Y., Qu, C., Li, Y., … Tian, X. (2022). Combined foliar application of zinc sulphate and selenite affects the magnitude of selenium biofortification in wheat (*triticum aestivum* L.). *food and energy Security, 11*(1), Article e342. https://doi.org/10.1002/FES3.342

Oumer, A., Joy, E. J. M., De Groote, H., Broadley, M. R., & Gashu, D. (2024). Burden of selenium deficiency and cost-effectiveness of selenium agronomic biofortification of staple cereals in Ethiopia. *British Journal of Nutrition, 132*(8), 1110–1122. https://doi.org/10.1017/S0007114524001235

Pasqualone, A., Vurro, F., Summo, C., Abd-El-Khalek, M. H., Al-Dmoor, H. H., Grgic, T., … Le-Bail, P. (2022). The large and diverse family of mediterranean flat breads: A database. *Foods, 11*(15), 2326. https://doi.org/10.3390/FOODS11152326

Passarelli, S., Free, C. M., Shepon, A., Beal, T., Batis, C., & Golden, C. D. (2024). Global estimation of dietary micronutrient inadequacies: A modelling analysis. *Lancet Global Health, 12*(10), e1590–e1599. https://doi.org/10.1016/S2214-109X(24)00276-6

Prom-u-thai, C., Rashid, A., Ram, H., Zou, C., Guilherme, L. R. G., Corguinha, A. P. B., … Cakmak, I. (2020). Simultaneous biofortification of rice with zinc, iodine, iron and selenium through foliar treatment of a micronutrient cocktail in five countries. *Frontiers in Plant Science, 11*, Article 589835. https://doi.org/10.3389/FPLS.2020.589835

Qiao, L., Zhang, X., Pi, S., Chang, J., Dou, X., Yan, S., … Xu, C. (2022). Dietary supplementation with biogenic selenium nanoparticles alleviate oxidative stress-induced intestinal barrier dysfunction. *Npj Science of Food, 6*(1), 1–17. https://doi.org/10.1038/S41538-022-00145-3

Ram, H., Naeem, A., Rashid, A., Kaur, C., Ashraf, M. Y., Singh Malik, S., … Cakmak, I. (2024). Agronomic biofortification of genetically biofortified wheat genotypes with zinc, selenium, iodine, and iron under field conditions. *Frontiers in Plant Science, 15*. https://doi.org/10.3389/fpls.2024.1455901

Rayman, M. P. (2012). Selenium and human health. *Lancet, 379*(9822), 1256–1268. https://doi.org/10.1016/S0140-6736(11)61452-9

Rigutto-Farebrother, J., & Zimmermann, M. B. (2024). Salt reduction and iodine fortification policies are compatible: Perspectives for public health advocacy. Nutrients 2024, vol. 16, page 2517, 16(15), 2517. Doi;https://doi.org/10.3390/NU16152517.

Rizzello, C. G., Nionelli, L., Coda, R., De Angelis, M., & Gobbetti, M. (2010). Effect of sourdough fermentation on stabilisation, and chemical and nutritional characteristics of wheat germ. *Food Chemistry, 119*(3), 1079–1089. https://doi.org/10.1016/J.FOODCHEM.2009.08.016

Roselli, C., Meli, M. A., Fagiolino, I., & Desideri, D. (2020). Bioaccessibility assessment of stable elements and 210Po in food. *PLoS One, 15*(8), Article e0236871. https://doi.org/10.1371/JOURNAL.PONE.0236871

Roselli, M., Finamore, A., Britti, M. S., & Mengheri, E. (2006). Probiotic bacteria bifidobacterium animalis MB5 and lactobacillus rhamnosus GG protect intestinal caco-2 cells from the inflammation-associated response induced by enterotoxigenic escherichia coli k88. *British Journal of Nutrition, 95*(6), 1177–1184. https://doi.org/10.1079/BJN20051681

Sánchez-Martínez, M., Pérez-Corona, T., Cámara, C., & Madrid, Y. (2015). Preparation and characterization of a laboratory scale selenomethionine-enriched bread. Selenium bioaccessibility. *Journal of Agricultural and Food Chemistry, 63*(1), 120–127. https://doi.org/10.1021/JF505069D

Shao, Y., Wolf, P. G., Guo, S., Guo, Y., Rex Gaskins, H., & Zhang, B. (2017). Zinc enhances intestinal epithelial barrier function through the PI3K/AKT/mTOR signaling pathway in caco-2 cells. *The Journal of Nutritional Biochemistry, 43*, 18–26. https://doi.org/10.1016/J.JNUTBIO.2017.01.013

Ulluwishewa, D., Anderson, R. C., McNabb, W. C., Moughan, P. J., Wells, J. M., & Roy, N. C. (2011). Regulation of Tight Junction Permeability by Intestinal Bacteria and Dietary Components1,2. *The Journal of Nutrition, 141*(5), 769–776. https://doi.org/10.3945/JN.110.135657

UNICEF. (2020). Nutrition, for Every Child: UNICEF Nutrition Strategy 2020–2030. www.unicef.org.

Wang, X., Valenzano, M. C., Mercado, J. M., Zurbach, E. P., & Mullin, J. M. (2013). Zinc supplementation modifies tight junctions and alters barrier function of CACO-2 human intestinal epithelial layers. *Digestive Diseases and Sciences, 58*(1), 77–87. https://doi.org/10.1007/S10620-012-2328-8

WHO. (2024). Global Iodine Nutrition Scorecard. https://ign.org/scorecard/.

Zhang, B., & Guo, Y. (2009). Supplemental zinc reduced intestinal permeability by enhancing occludin and zonula occludens protein-1 (ZO-1) expression in weaning piglets. *British Journal of Nutrition, 102*(5), 687–693. https://doi.org/10.1017/S0007114509289033

Zhou, S., Zhang, D., Kong, Y., Zhang, Q., & Cui, X. (2024). In vivo bioavailability and in vitro bioaccessibility of iodine in edible seaweeds: Method development and health implications. *Environmental Science & Technology, 58*(51). https://doi.org/10.1021/ACS.EST.4C08990

Zou, C., Du, Y., Rashid, A., Ram, H., Savasli, E., Pieterse, P. J., … Cakmak, I. (2019). Simultaneous biofortification of wheat with zinc, iodine, selenium, and iron through foliar treatment of a micronutrient cocktail in six countries. *Journal of Agricultural and Food Chemistry, 67*(29), 8096–8106. https://doi.org/10.1021/acs.jafc.9b01829