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Digestive Behavior of Minerals in Infant Formulas: An In Vitro Study of Casein Phosphopeptides, 1,3-Dioleoyl-2-Palmitoylglycerol, and Lactoferrin Fortification

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Abstract— *The bioavailability of minerals in breast milk (BM) and infant formula (IF) plays a crucial role in infant nutrition and development. This study investigated the effects of casein phosphopeptides (CPP), 1,3-dioleoyl-2-palmitoyl-glycerol (OPO), and lactoferrin (LTF) on mineral content and bioaccessibility following in vitro gastrointestinal digestion. In vitro dynamic digestion was performed, and the mineral content was quantified using inductively coupled plasma mass spectrometry (ICP-MS). The results revealed that the combination of CPP, OPO, and LTF significantly increased the bioaccessibility of calcium (Ca), magnesium (Mg), zinc (Zn), and iron (Fe) compared to that in the control sample. The bioaccessibility values are 12.4–20.7% (Ca), 11.3–55.2% (Fe), 13.6–28.2% (Mg), and 12.7–25.6% (Zn). These findings suggest that incorporating CPP, OPO, and LTF into infant formulas may enhance mineral bioavailability, potentially supporting better mineral absorption and improving infant nutrition.*

Keywords— ***Breast milk, digestion, infant formula, minerals, bioaccessibility***

I. INTRODUCTION

Minerals play critical roles in infant growth, bone development, immune function, and metabolic processes. Essential minerals, such as calcium (Ca), magnesium (Mg), iron (Fe), and zinc (Zn), contribute to skeletal formation, enzymatic activity, oxygen transport, and immune defense [1]. Their bioavailability depends not only on dietary intake but also on interactions with other food components during digestion [2]. Breast milk (BM) is considered the gold standard for infant nutrition, providing minerals in highly bioavailable forms [3]. This superior bioavailability is attributed to the specific bioactive components present in BM, including casein phosphopeptides (CPPs), structured lipids such as 1,3-dioleoyl-2-palmitoyl-glycerol (OPO), and lactoferrin (LTF). However, replicating this bioavailability in infant formula (IF) remains challenging because of compositional and structural differences [4].

Casein phosphopeptides (CPPs) are phosphorylated peptides released during casein digestion, characterized by their ability to bind divalent cations like calcium [5]. The formation of soluble CPP-mineral complexes prevents mineral precipitation at alkaline pH, facilitating their intestinal absorption [6]. For instance, research has shown that CPP supplementation improves Ca absorption in infants and adults [7]. CPPs' protective effect of CPPs on minerals during digestion makes them promising ingredients for formula fortification. 1,3-dioleoyl-2-palmitoyl-glycerol (OPO), a structured lipid designed to mimic the triglyceride composition of human milk fat, also influences mineral absorption in infants. In BM, palmitic acid is predominantly esterified at the sn-2 position, which promotes Ca absorption by minimizing the formation of insoluble calcium soaps in the intestine [8]. A previous study has shown that sn-2-palmitate-enriched

formula feeding promotes weight gain and bone mineral accumulation and reduces stool fatty acid soap content compared to the standard formula. No significant differences in these outcomes were observed when compared with human milk [9]. Lactoferrin (LTF) is an iron-binding glycoprotein abundantly present in BM, playing a multifaceted role in Fe metabolism and immune function. It facilitates Fe absorption by binding free iron and maintaining it in a soluble form suitable for uptake in the intestine [10]. By maintaining Fe in a soluble state, LTF enhances its bioavailability and exerts antimicrobial effects by depriving pathogenic bacteria of iron [11]. Studies have shown that lactoferrin-supplemented formulas improve the Fe status in infants without causing adverse gastrointestinal effects [12].

Although the individual effects of CPP, OPO, and LTF on mineral absorption are well documented, their combined impact remains unclear. The interaction of CPP with minerals, combined with the structural benefits of OPO and iron-binding properties of LTF, may exert synergistic effects on mineral bioavailability. This study aimed to investigate the influence of these components on mineral content and bioaccessibility following in vitro gastrointestinal digestion. Using ICP-MS, we compared the mineral profiles of BM, a control formula (CF), two formulas containing different combinations of CPP, OPO, and LTF, and one formula containing OPO and LTF without CPP. These findings provide insights into optimizing formula composition to improve mineral bioavailability and better mimic the nutritional properties of BM.

II. MATERIAL AND METHODS

2.1. Materials

2.1.1. Samples

Five samples were analyzed in this study, including BM collected from 10 healthy lactating mothers in Changsha, Hunan Province, between 1 and 9 months postpartum. Milk was obtained using an electric breast pump in the morning, with 30 mL aliquots stored at −80°C and transported on dry ice to Jiangnan University for analysis. Informed consent was obtained from all participants, and the study was approved by the Medical Ethics Committee of Jiangnan University (ethics approval number: JNU20220901IRB15, Wuxi, Jiangsu, China). Mothers with a history of smoking, alcohol or drug use, infectious diseases, cancer, or other chronic conditions were excluded from the study. All other samples were stage 3 formulas, including CF, a commercially available standard formula, and three experimental formulas, TF1, TF2, and TF3, provided by Ausnutria Dairy (China) Co., Ltd. The composition of the formulas is presented in Table 1. All samples were prepared according to the manufacturer's guidelines, homogenized, and subjected to in vitro gastrointestinal digestion. BM samples were thawed at 4°C and mixed gently to maintain homogeneity before analysis.

Table 1. Infant Formula Composition

Milk samples	OPO (g/100g)	CPP (mg/100g)	LTF (mg/100g)
CF	-	-	-
TF1	4.1	-	100
TF2	4	166	65
TF3	4.1	200	65

2.1.2. Chemicals

All chemicals used in this study were of analytical grade and supplied by Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Enzymes for simulating gastrointestinal digestion were procured from Sigma-Aldrich Co. (St. Louis, MO, USA), including pepsin (P7125, from porcine gastric mucosa) for gastric digestion and pancreatin (P7545, from porcine pancreas) along with bile salts (48305) for intestinal digestion. Enzyme solutions were freshly prepared to preserve optimal activity during digestion. Nitric acid (HNO_3, trace metal grade) and hydrochloric acid (HCl, trace metal grade), were obtained from Merck KGaA (Darmstadt, Germany).

2.2. Methods

2.2.1. In-vitro Dynamic Digestion

The simulated digestion solution was prepared using the appropriate electrolyte stock solutions, enzymes, calcium chloride ($CaCl_2$), and water, following the method described by M. Minekus with minor modifications [13]. All digestive fluids, including 0.5 M HCl and 1 M $NaHCO_3$, were preheated and maintained at 37°C using the integrated water bath of the Human Stomach-Intestine System IV (DHSI-IV), developed by Xiao Dong Pro-health (Suzhou) Instrumentation Co., Ltd. (Suzhou, China). This system replicates gastrointestinal conditions by simulating physiological processes, such as pH variations, enzymatic

activity, and peristaltic movements, in a dynamic controlled environment.

For the gastric phase, pepsin and lipase were dissolved in simulated gastric fluid (SGF), which contained 7.80 mmol/L K^+, 0.90 mmol/L $H_2PO_4^-$, 25.50 mmol/L HCO_3^-, 70.20 mmol/L Cl^-, 0.10 mmol/L Mg^{2+}, 1.00 mmol/L NH_4^+, 72.20 mmol/L Na^+, and 0.15 mmol/L Ca^{2+}, with the pH adjusted to 3.2. The final volume of the SGF was set to 100 mL, resulting in pepsin activity of 400 U/mL and gastric lipase activity of 120 U/mL. Digestion progressed continuously to the intestinal phase, during which pancreatic enzymes and bile salts were dissolved in simulated intestinal fluid (SIF). The SIF contained 7.60 mmol/L K^+, 2.80 mmol/L $H_2PO_4^-$, 85 mmol/L HCO_3^-, 55.50 mmol/L Cl^-, 0.33 mmol/L Mg^{2+}, 123.40 mmol/L Na^+, and 0.60 mmol/L Ca^{2+}, with the pH adjusted to 6.5. The final volume of the SIF was adjusted to 100 mL, yielding a pancreatic enzyme concentration of 20 U/mL and a bile salt concentration of 10 mM. Milk samples (125 mL) diluted to a protein concentration of 10 mg/mL were introduced into the digestion chamber to initiate gastrointestinal digestion. The process continued for 120 min without interruption, mimicking the continuous transit of food through the gastric and intestinal phases. Samples were collected at 0 min (before digestion) and subsequently at 120 min to monitor the dynamic changes in mineral content and bioaccessibility. After collection, the samples were centrifuged at 10,000 × g for 30 min at 4°C to separate the bioaccessible (soluble) and non-bioaccessible (insoluble) fractions. The supernatant was stored at −80°C until further analysis.

2.2.2. ICP-MS Mineral Determination

The mineral content of the digested samples was determined using Inductively Coupled Plasma Mass Spectrometry (ICP-MS). For sample preparation, 3 mL of the sample was mixed with 4.5 mL of concentrated nitric acid (HNO_3, trace metal grade) in a digestion tube and allowed to digest overnight. After complete digestion, the mixture was diluted to 50 mL with ultrapure water in a volumetric flask, thoroughly mixed, and transferred to a 10–15 mL centrifuge tube for analysis. The solution was then filtered through a syringe filter into a clean centrifuge tube to obtain 3–5 mL of the clear filtrate. The filtrate was further diluted 10-fold, 100-fold, and 1000-fold as needed for subsequent mineral analysis. ICP-MS analysis was performed using a Thermo Fisher iCAP Q ICP-MS system (Thermo Fisher Scientific, Waltham, MA, USA). The ICP-MS was operated under the following conditions: RF power: 1550 W, plasma gas flow rate: 15 L/min, nebulizer gas flow rate: 1.05 L/min, collision/reaction gas: helium (He), and dwell time: 100 ms per isotope.

2.3. Statistical Analysis

All experimental data were analyzed using SPSS Statistics version 27.0. The results are expressed as the mean ± standard deviation (SD) from three independent replicates. Comparisons between groups were performed using one-way analysis of variance (ANOVA), with $p < 0.05$ considered statistically significant. All graphs were generated using OriginPro 2024 software.

III. RESULTS AND DISCUSSION

3.1. Calcium Content

Ca retention varied significantly among the milk samples ($p < 0.0.5$), highlighting the impact of compositional differences on mineral solubility during digestion (Fig. 1). BM, which had the lowest initial calcium concentration (252.36 ± 12.23 mg/L), retained a relatively high amount of calcium post-digestion (49.49 ± 11.05 mg/L), reflecting its superior natural bioavailability. This finding is consistent with that of previous studies [3]. In contrast, CF, which lacked bioactive components, initially contained the highest calcium concentration (887.23 ± 13.25 mg/L) but retained only 109.71 ± 2.85 mg/L after digestion, suggesting significant precipitation at intestinal pH. Among the experimental formulas, TF1 had a lower initial calcium concentration (669.4 ± 131.3 mg/L) than CF but retained 92.22 ± 2.99 mg/L, suggesting that OPO helped to prevent calcium precipitation. TF2 demonstrated the highest Ca retention (129.71 ± 7.81 mg/L) despite starting with a lower concentration (626.78 ± 114.9 mg/L), likely due to the presence of CPP in the formulation. TF3, which contained the highest CPP concentration, started with the lowest calcium content (362.54 ± 45.16 mg/L) and retained 51.54 ± 8.03 mg/L after digestion. Although CPP enhances calcium solubility, its effectiveness depends on the initial calcium concentration, as observed in previous studies [14]. Overall, these results confirm that OPO prevents calcium precipitation, whereas CPP enhances retention, particularly when sufficient calcium is available, supporting their role in improving the formula composition to better mimic human milk.

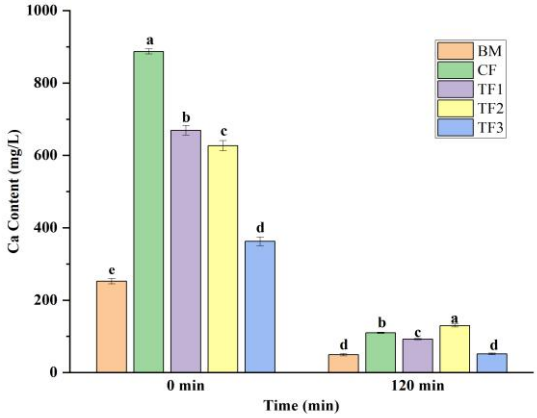

Fig. 1 Changes in Calcium Content Before and After In-Vitro Gastrointestinal Digestion

3.2. Magnesium Content

Mg is a vital mineral involved in over 300 enzymatic reactions, neuromuscular function, and bone development, making its bioavailability crucial for infant growth [15]. BM is recognized for its superior magnesium bioavailability due to its association with casein micelles and citrate, which help maintain solubility during digestion. The changes in Mg content before and after gastrointestinal digestion are presented in Fig. 2. In this study, BM, despite having the lowest initial magnesium concentration (29.29 ± 1.29 mg/L), retained 8.25 ± 0.12 mg/L post-digestion, supporting previous findings that magnesium in human milk remains highly bioavailable owing to its interaction with organic ligands [16]. In contrast, CF, which initially contained a significantly higher magnesium concentration (89.78 ± 3.15 mg/L), retained only 16.05 ± 1.68 mg/L ($p < 0.05$), indicating significant precipitation under intestinal conditions and reduced solubility compared with BM. TF1 had the highest initial magnesium content (107.3 ± 13.32 mg/L) but retained only 14.6 ± 2.32 mg/L, demonstrating no significant improvement over the CF. In contrast, TF2 exhibited the highest Mg retention despite a lower initial content (86.01 ± 2.75 mg/L). TF3, despite containing the highest CPP concentration, had a lower initial magnesium content (56.99 ± 7.96 mg/L) and retained 9.61 ± 1.01 mg/L, showing lower magnesium retention than that of TF2. This suggests that while CPP enhances magnesium solubility, its effectiveness depends on the initial mineral concentration and its interactions with other formula components. Previous studies have reported that Mg absorption is influenced by the presence of chelating peptides; however, its retention is also affected by intestinal pH and competing mineral interactions [17,18]. These findings highlight that CPP plays a key role in magnesium solubility; however, optimal fortification strategies must consider the initial mineral concentrations to maximize the absorption efficiency.

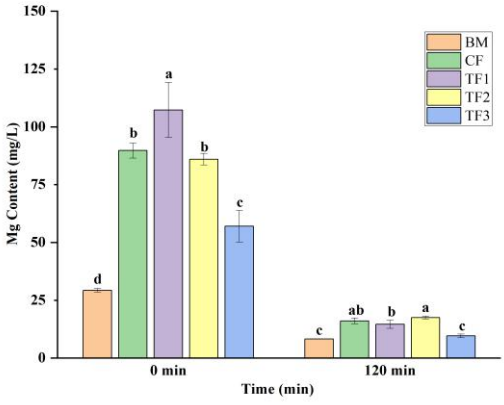

Fig. 1 Changes in Magnesium Content Before and After In-Vitro Gastrointestinal Digestion

3.3. Zinc Content

Zn is a critical micronutrient essential for immune function, growth, and enzymatic activity in infants [19]. Human milk is recognized for its highly bioavailable zinc, which is bound to casein and low-molecular-weight ligands, preventing its precipitation during digestion [20]. The changes in Zn content before and after gastrointestinal digestion are presented in Fig. 3. In this study, BM had an initial zinc concentration of 1577.53 ± 153.45 mg/L, with 404.67 ± 41.25 mg/L remaining after digestion. A significant loss of zinc during digestion is expected, as free zinc tends to precipitate at higher pH levels during the intestinal phase. CF, with a considerably higher initial Zn concentration (5660.12 ± 219.59 mg/L), retained only 803.54 ± 0.6 mg/L, indicating that a substantial portion of Zn was lost after digestion. Among the test formulas, TF1 initially contained 5075.41 ± 731.22 mg/L but retained only 695.02 ± 151.73 mg/L, showing no significant improvement over CF ($p < 0.05$). However, TF2 demonstrated the highest Zn retention (1267.51 ± 149.76 mg/L), despite its lower initial concentration (5682.19 ± 257.6 mg/L). Conversely, TF3, which contained the highest CPP concentration, started with 5603.14 ± 532.22 mg/L but retained only 710.65 ± 157.6 mg/L after digestion. Overall, these findings confirm that OPO, CPP, and LTF synergistically enhance Zn solubility, making them valuable components for improving mineral bioavailability in infant formulas.

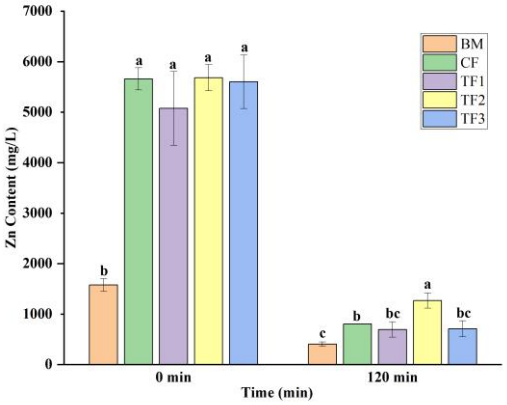

Fig. 1 Changes in Zinc Content Before and After In-Vitro Gastrointestinal Digestion

3.4. Iron Content

Fe is a critical micronutrient required for oxygen transport, cognitive development, and immune function in infants [21]. However, its bioavailability is often limited because of its tendency to form insoluble precipitates at intestinal pH or bind to dietary inhibitors [22]. The changes in Fe content before and after gastrointestinal digestion are presented in Fig. 4. BM exhibited a lower initial iron concentration (429.73 ± 21.68 mg/L) than the formula samples but retained a substantially higher proportion after digestion (237.18 ± 6.86 mg/L). This superior retention is attributed to LTF, an iron-binding glycoprotein that protects Fe from oxidation and enhances its intestinal uptake by binding to specific receptors in the infant gut, as previously reported in studies on BM composition [23]. CF had the highest initial iron concentration but retained only 1,397.03 ± 181.26 mg/L post-digestion, indicating significant Fe loss. This suggests that, in the absence of bioactive iron-binding proteins, iron precipitates more readily, leading to reduced bioaccessibility. TF1 showed a similar trend, suggesting that OPO alone did not significantly improve iron retention. In contrast, TF2 demonstrated the highest Fe retention among the formulas (1,887.87 ± 253.73 mg/L), despite a lower initial Fe content (13,350.23 ± 416.14 mg/L). This suggests that the combination of LTF and CPP plays a crucial role in maintaining the Fe solubility. Interestingly, TF3, which contained the highest CPP concentration, retained less Fe (1,355.24 ± 770.24 mg/L) than TF2, despite its similar initial iron content (12,059.3 ± 1412.95 mg/L). This suggests that excess CPP may not proportionally enhance Fe bioaccessibility, possibly due to competitive mineral binding or interactions that affect Fe solubility. Similar findings have been reported by Petry et al. (2016), who observed that high doses of dietary peptides do not always improve Fe absorption and may interfere with mineral interactions [24]. This supports the notion that optimizing formula composition, rather than simply increasing fortification levels, is essential for improving iron bioavailability in infant nutrition [25].

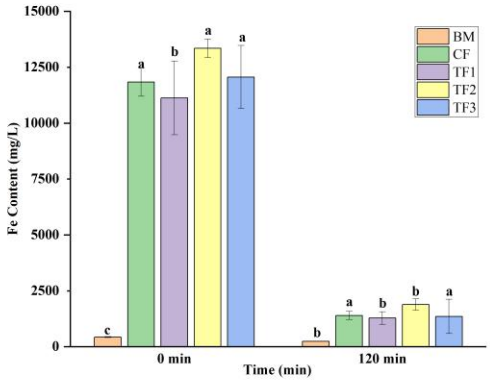

Fig. 1 Changes in Iron Content Before and After In-Vitro Gastrointestinal Digestion

3.5. Mineral Bioaccessibility

Mineral bioaccessibility, which refers to the fraction of a nutrient that remains soluble and available for absorption after digestion, plays a critical role in determining the nutritional efficacy of IF [26]. In the present study, the bioaccessibility of Ca, Mg, Zn, and Fe was determined, and the results are shown in Table 2. The Ca bioaccessibility values ranged from 12.4% to 20.7%. Mg showed higher bioaccessibility than Ca, with BM at 28.2% and TF2 at 20.4%, likely due to its lower precipitation. Mg is known to be bioavailable and well utilized by animals (approximately 80%), i.e these elements are more soluble under gastrointestinal conditions [27]. Thus, these Mg bioaccessibility values are higher than those of Ca. The bioaccessibility values for Fe and Zn were, on average, higher than those for the other elements. It is well known that the bioaccessibility of essential minerals is highly dependent on the food matrix.

Table 2. Bioaccessibility of the Minerals from the Breast Milk and Infant Formulas

Samples	Ca (%)	Mg (%)	Zn (%)	Fe (%)
BM	19.60±0.82^{a}	28.17±0.66^{a}	25.62±0.61^{a}	55.22±2.43^{a}
CF	12.36±0.10^{c}	17.90±1.19^{bc}	14.21±0.54^{b}	11.77±0.90^{b}
TF1	13.78±0.04^{b}	13.65±1.53^{d}	13.72±2.58^{b}	11.42±0.86^{b}
TF2	20.70±0.22^{a}	20.38±0.17^{b}	22.26±1.63^{a}	14.13±1.74^{b}
TF3	14.22±0.66^{b}	16.94±1.68^{b}	12.58±1.62^{b}	10.84±5.15^{b}

[1]*The results are expressed as mean ± standard deviation (n=3)*

[2] *Mean values with different letters differ significantly at p <0.05.*

BM demonstrated the highest bioaccessibility across all measured minerals, consistent with prior research indicating that BM contains naturally optimized mineral-binding components that enhance mineral solubility and absorption [28]. Among the formula samples, TF2 consistently exhibited the highest bioaccessibility for all four minerals, surpassing CF, TF1, and TF3 values ($p < 0.05$). Specifically, TF2 demonstrated the highest bioaccessibility for Ca (20.7%), Mg (20.38%), Fe (14.13%), and Zn (22.26%), indicating that the combination of CPP, OPO, and LTF significantly improved mineral solubility. This finding is supported by a finding, which reported that structured lipids, such as OPO, enhance Ca absorption by reducing Ca soap formation, whereas CPP improves divalent cation retention [29]. Finally, the bioaccessibility of minerals in IFs is lower than that in BM. However, the high bioaccessibility values for the IFs studied in the present work can be considered satisfactory, since the elements are quite soluble; therefore, it could result in suitable absorption by the infants. These findings highlight the importance of optimizing CPP, OPO, and LTF concentrations to enhance mineral bioavailability in IF, bridging the gap between human milk and formula nutrition.

IV. CONCLUSION

This study demonstrates that casein phosphopeptides (CPP), structured lipids (OPO), and lactoferrin (LTF) enhance

mineral retention and bioaccessibility in infant formulas (IFS), helping to better mimic breast milk (BM). BM exhibited the highest mineral bioaccessibility, whereas TF2 showed the greatest retention of calcium, magnesium, iron, and zinc among the formula samples, confirming the synergistic role of these bioactive components. CPP improved the solubility of calcium, magnesium, and zinc; LTF enhanced iron bioavailability; and OPO contributed to calcium retention by reducing precipitation. Despite TF3 containing a higher CPP concentration than TF2, its slightly lower mineral bioaccessibility suggests that excessive CPP levels may interfere with mineral interactions, emphasizing the need for optimized fortification rather than excessive supplementation of the product. These findings support the inclusion of CPP, OPO, and LTF in IF to enhance mineral absorption, though further in vivo studies and clinical trials are needed to confirm their long-term benefits for infant growth and development.

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