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How α -lactalbumin and β –casein level in infant formula influence the protein and minerals absorption properties by using Caco-2 cell model

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ABSTRACT

This study investigated the differences in absorption of infant formula with different levels of α-lactalbumin (α-La) and β-casein (β-CN), and how α-La and β-CN (α+β) affect mineral absorption using the Caco-2 cell model. Compared to the control group, the free amino acid content transported to the basal side significantly increased in the fortification of α-La and β-CN, particularly in the α+β (high) group, which reached 11.343 μmol/L. Furthermore, fortification of α-La and β-CN upregulated the expression of the amino acid transporter SLC6A14 and the oligopeptide transporter PEPT1, in particular the expression of SLC6A14 in the α+β (high) group was 103% of that of the control group. In addition, fortification of α-La and β-CN enhanced the absorption of Ca, Mg, and Zn. The absorption rates of Ca, Mg, and Zn in α+β (high) group increased to 31.27%, 13.66%, and 58.59%, respectively, of which α-La contributed the most on Ca absorption. In conclusion, α-La and β-CN fortification not only increased the absorption of amino acids and peptides but also promoted the absorption of minerals, which provide evidence for the benefits of adjustment of α-La and β-CN in the infant formula.

1. Introduction

Although breast milk is the best food for babies, the breastfeeding rate in the first six month is still quite low, which was around 44% all over the word (UNICEF, 2020). This indicates that certain number of babies have to rely on infant formula to meet their growth and development. Infant formula is usually produced by using breast milk as standard and bovine or caprine milk as milk source with the addition of ingredient. Although dairy industry had tried their best to mimic the breast milk, there are still gaps between breastfeeding and infant formula feeding in the short-term and long-term health of babies. These cover a broad spectrum of immediate effects, such as the maturation of intestinal epithelium (Holscher, Bode, & Tappenden, 2017; Suárez et al., 2019) and the immune system in the newborns (Chen et al., 2020; Ma et al., 2020; van Dongen, Ioannou, Wesseling, Beekmann, & Belzer, 2022), strong serum antioxidant capacity (Alpinar, Torun, Özkaya, Uzuner, & Erenberk, 2012) and gastrointestinal health (Fang, Lian, Yang, Duan, & He, 2021; Rajesh et al., 2023), particularly in countries with lower income. These differences might be related to the differences in the composition between breast milk and infant formula.

Among those composition in infant formula, protein is one of the most important nutrients as it is the cornerstone of life activities. The ratio of whey to casein is the most significant difference between breastmilk and infant formula. This ratio has been optimized since 1962 (Happe & Gambelli, 2015). However, the subcomponents in whey and casein still differ between them. α-lactalbumin (α-La) is the most abundant whey protein in breast milk, which can promote mineral absorption and provides essential amino acids for maintaining health (Tinghäll Nilsson et al., 2023; Yiğit, Bielska, Cais-Sokolińska, & Samur, 2023), as it contains all kinds of essential amino acids (Layman et al., 2018), especially tryptophan (Trp). In contrast, β-lactoglobulin (β-Lg) is the most abundant in infant formula. In breast milk, β-casein (β-CN) is the most abundant protein. Upon digestion, it provides essential amino acids and bioactive peptides (Wada, Phinney, Weber, & Lönnerdal,

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Table 1
The percentage of α-lactalbumin and β-casein in the total protein of the materials.

	Control	α+β (low)	α+β (high)	α-La	β-CN
α-La %	10.00	20.97	29.03	–	99.35
β-CN %	14.00	26.03	29.00	99.42	–

Note: α+β (low) with fortified low ratio of α-lactalbumin and β-casein; α+β (high) with fortified high ratio of α-lactalbumin and β-casein; α-La with pure α-lactalbumin protein and β-CN with pure β-casein protein.

Table 2
Parameters of target gene and β-actin.

	Forward Primer	Reverse Primer
SLC6A19	CATGGTGGGACTGTATTACAACA	CTCGTCCACATACCCTGTCTG
SLC6A14	ACCGTGGTAACTGGTCCAAAA	CGCCTCCACCATTGCTGTAG
SLAlC1A1	TTTCTGTACCACTCTCATTGCTG	TCCACCGTACTGACTTCAGGG
Pept1	GACAAGCAGTCACCTCAGTAAG	AGTCCCGAGAGCTATCAGGG
Occludin	TCTGGCTATTTTAGTTGCCACAG	AGAGAGCCTGACCAAATTCGT
Claudin-2	CGGGACTTCTACTCACCACTG	GGATGATTCCAGCTATCAGGGA
Claudin-3	CTGCTCTGCTGCTCGTGTC	CGTAGTCCTTGCGGTCGTAG
Zo-1	ACCAGTAAGTCGTCCTGATCC	TCGGCCAAATCTTCTCACTCC
Zo-2	GGCCTACGACCCAGACTAC	ACTCTTCGTTCGCTCTGCTTT
β-actin	GGACCTGACTGACTACCTCAT	CGTAGCACAGCTTCTCCTTAAT

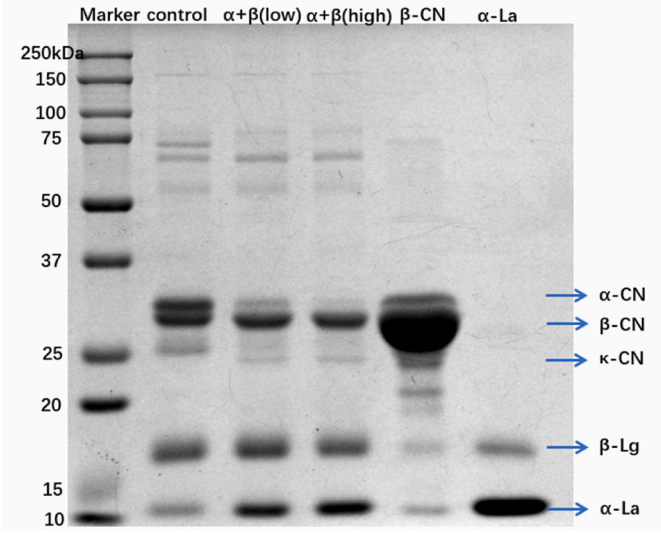

Fig. 1. Reducing SDS-PAGE analysis of samples. α+β (low) with fortified low ratio of α-lactalbumin and β-casein; α+β (high) with fortified high ratio of α-lactalbumin and β-casein; α-La with pure α-lactalbumin protein and β-CN with pure β-casein protein.

2017). In contrast, infant formula contains αs_1-casein.

Proteins are hydrolysed into small peptides and amino acids by gastrointestinal enzymes. They are then transported into the body by small intestinal epithelial cell transport carriers. These nutrients are absorbed by the body to promote good health. The effects of amino acids on infant growth include supporting cell growth (Hosios et al., 2016), brain development (Yu et al., 2022) and hormone secretion (Flynn, Shaw, & Becker, 2020; Tsugawa, Handa, & Imai, 2019). At the same time, bioactive peptides also play an important role in the body, such as promoting mucosal growth, enhancing immune function, anti-inflammatory, anti-obesity and so on (Bouzerzour et al., 2012; Chen et al., 2021). Additionally, dairy protein contributes to the absorption of minerals such as Ca, Mg, and Zn, which are crucial for the development of bones, teeth, and the immune system, as well as the intellectual development of infants (Cormick & Belizán, 2019; Kelleher, Chatterton, Nielsen, & Lönnerdal, 2003). Protein, such as α-La and β-CN, contains plasma binding sites of minerals (Chamani, 2010; Rodzik et al., 2023), and mineral cations have strong binding ability with some amino acid groups in proteins (such as COO^- group), which is beneficial to the absorption of ions into the body (Gołębiowski et al., 2022; Li, O'Mahony, Kelly, & Brodkorb, 2020; Mizuguchi, Nara, Kawano, & Nitta, 1997; Permyakov, 2020; Rodzik et al., 2023; Walters, Esfandi, & Tsopmo, 2018). Peptides produced by hydrolysis of α-La and β-CN may still retain the binding sites of mineral ions. For instance, casein phosphopep Rodzik tides contain Ca^{2+} binding sites that combine with Ca^{2+} to form soluble substances. This prevents Ca from forming calcium phosphate precipitates in the neutral or alkaline environment of the small intestine, thereby promoting Ca absorption (Sun et al., 2016; Wang, Bai, Wang, Wang, & Wang, 2023).

Previous studies have compared the digestibility and absorption of infant formula with different protein compositions and their influence on the absorption of minerals (Chauvet et al., 2023; Halabi et al., 2022; Lambers, Wissing, & Roggekamp, 2023; Lin, Ouyang, Luo, & Ye, 2023). As the contents of α-La and β-CN in infant formula increasing, the phenomena and digestive products were different during digestion on the basis of whey:casein = 60:40 (Rafiee-Tari, Fan, Archbold, Arranz, & Corredig, 2019; Tari et al., 2018). Our previous studies had explored the digestion patterns of infant formula fortified with both α-La and β-CN and found that the more closer the ratio to breast milk,the more similar was the digestion pattern (Huang, Zhang, Lan, & Zhou, 2022). The protein digestion pattern may subsequently influence the absorption and in turn for the fate of minerals. Davis A M et al. found that the plasma amino acids and growth status of infants fed with α-La-rich infant powder were similar to those of breast-fed infants (Davis, Harris, Lien, Pramuk, & Trabulsi, 2008; Trabulsi et al., 2011). Silvina R adjusted the ratio of casein to whey in formula milk to be similar to that in breast milk. The absorption of Zn was significantly higher in the adjusted milk compared to unadjusted milk, indicating that adjusting the protein composition affects mineral absorption (Fioravanti, Milani, de Paiva, & Morgano, 2020). However, it is still unknown how fortifying both α-La and β-CN impacts the absorption rate and fate of minerals in infant formula.

The objective of this study was to investigate the absorption characteristics of infant formula enriched with different levels of α-La and β-CN under the condition of whey:casein = 60:40, using the Caco-2 cell model. Additionally, we examined the absorption characteristics of pure α-La and pure β-CN. We also investigated how the fortification of α-La and β-CN in infant formula affected mineral absorption by the cell model.

2. Materials and methods

2.1. Materials

2.1.1. Reagents

Pepsin, gastric lipase, and pancreatin were purchased from Sigma Aldrich Co. (St. Louis, MO, USA). Fetal bovine serum, trypsin-EDTA and high glucose DMEM medium were purchased from Gibco (Big Island, New York, USA). Transwell insert plates were came from Corning (Corning, New York, USA). The ELISA kit was purchased from Shanghai Enzyme-linked co., Ltd (Shanghai, China). Caco-2 cells were obtained from the Cell Bank of the Type Culture Collection Committee of China Academy of Sciences (Shanghai, China). α-La was purchased powder from Agropur (Quebec, Canada). Fresh milk was provided by Tianzi Dairy Co., Ltd (Wuxi, Jiangsu, China). The other ingredients used in the study were of analytical grade obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

2.1.2. Sample preparation

The dosage of each raw material in the strengthening group were shown in Table 1. Control, α+β (low) and α+β (high) groups belong to

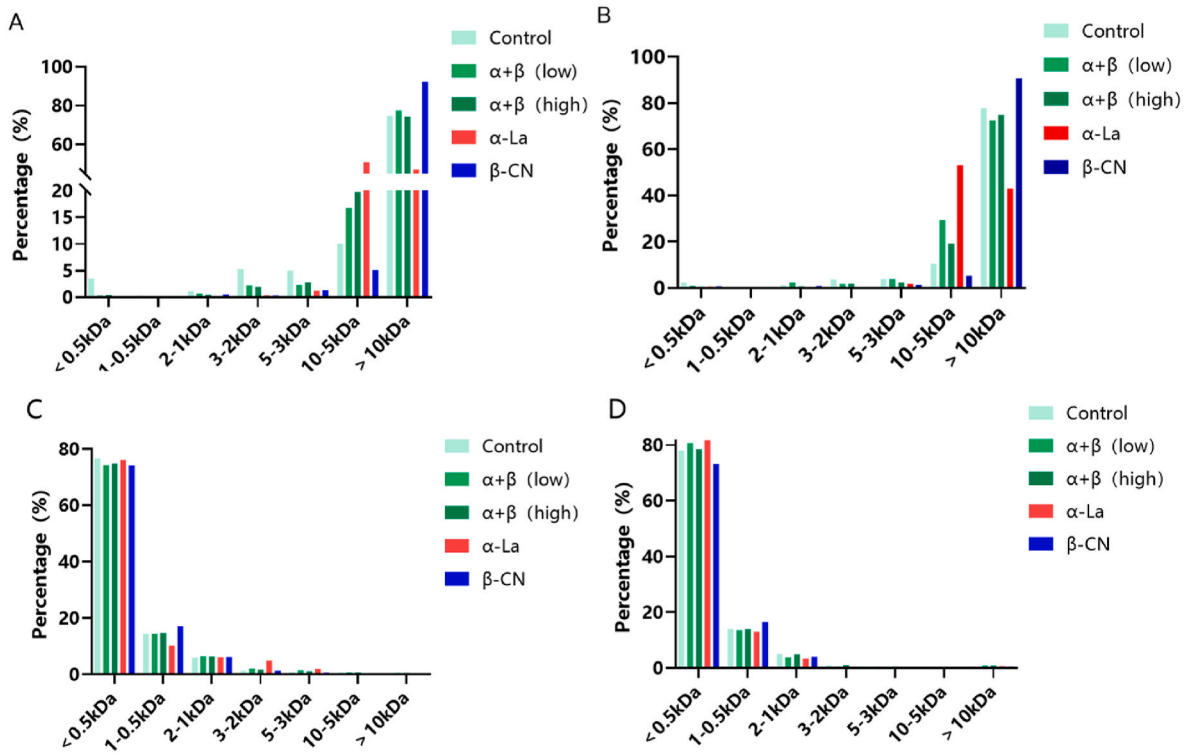

Fig. 2. Polypeptide molecular weight distribution of the digesta obtained from control, α+β (low), α+β (high), α-La, β-CN. α+β (low) with fortified low ratio of α-lactalbumin and β-casein; α+β (high) with fortified high ratio of α-lactalbumin and β-casein; α-La with pure α-lactalbumin protein and β-CN with pure β-casein protein. (A: G0; B: G0 samples with minerals added; C: I120; D: I120 samples with minerals added).

mixed protein system, which contains α-CN, β-CN, β-lg, α-La and other components.

2.2. Methods

2.2.1. Exacting β-CN

β-CN was extracted from milk using a series of steps. Fresh milk was centrifuged to obtain skim milk. The pH was then adjusted to 4.6 to obtain a casein precipitate, which was subsequently re-dissolved and the pH adjusted to 11.0. A $CaCl_2$ solution was added, and the pH was adjusted to 7.0 before centrifuging to obtain a precipitate containing αs-CN and β-CN. The precipitate was dissolved at 25 °C, and the pH was adjusted to 4.6. The mixture was stirred overnight at 4 °C before being centrifuged to obtain a supernatant containing β-CN. The pH of the supernatant was adjusted to 4.6, followed by stirring at 35 °C for 20 min. The resulting β-CN precipitate was obtained by centrifugation at 25 °C and subsequently freeze-dried for storage.

2.2.2. SDS-PAGE analysis

A 12% separation gel and a 4% concentrated gel were made at room temperature (25 °C). Next, 100 μL of the undigested diluted sample solution and gastric digestive juice were mixed with 2 × sample buffer (containing 5% β-mercaptoethanol) in equal volumes. The mixture was then heated in boiling water for 3 min. After cooling, 5 μL was taken and the gel electrophoresis was run with 60 V for the separation gel and 120 V for the concentrated gel. After electrophoresis, the rubber plate was stained with a Coomassie brilliant blue dye solution for 2 h. It was then decolorised with a decolorising solution until the background became transparent. For gastrointestinal digestion, the remaining steps were the same as those for gastric digestion, except that undigested samples were diluted fourfold, gastric digestion samples were diluted twofold, and intestinal digestive juice was not treated. Furthermore, the sample volume was changed to 10 μL.

2.2.3. In vitro gastrointestinal digestion

The gastrointestinal digestion procedure was performed as described previously (Huang et al., 2022; Ying, Yanmei, Kui, Lina, & Peng, 2021). The solution's protein concentration was adjusted to 10 mg/mL. The pH was then adjusted to 4.0, and the sample was digested with porcine pepsin at a ratio of 22.75 U pepsin per mg of milk protein at 37 °C for 120 min. To stop the pepsin decomposition, the pH was adjusted to 7.0. The gastric digest was supplemented with an equal volume of simulated intestinal juice (SIF) containing 50 mmol/L KH_2PO_4, 20 mmol/L $CaCl_2$, and pH 6.5 adjusted with 1 mol/L NaOH. The final concentrations of the components were as follows: 8.63 U/mL of trypsin, 0.1 U/mL of α-chymotrypsin, and 1.32 mg/mL of bile salts. The mixture was then digested at 37 °C for 120 min and the reaction was stopped by heating the mixture at 95 °C for 5 min.

2.2.4. Caco-2 cell culture and treatment

The cells were incubated in a 37 °C and 5% CO_2 incubator, and the medium was changed every two days. Once the cells reached 90% confluence, they were trypsinized with 0.25% (v/v) trypsin-EDTA and then inoculated into new T75 culture bottles or Transwell plates (Corning, California, USA), depending on the experimental requirements. The cell survival rate was determined using CCK-8 (Long et al., 2019). Undiluted samples were selected for treatment because the samples at each concentration were not toxic to cells.

2.2.5. Permeability coefficient of fluorescein sodium

When the transmembrane potential reached 400 Ω cm^2, the permeability coefficient was measured using sodium fluorescein. The old culture medium was removed and the cell monolayer was washed three times with Hanks equilibrium solution. Then, 400 μL of 1 mg/mL fluorescein sodium solution were added to the upper chamber and 1 mL of Hanks equilibrium solution was added to the lower chamber. The lower layer liquid was extracted at 0.5, 1.0, 1.5, 2.0 and 2.5 h and the absorbance were measured at 490 nm. Standard solutions of 0.5, 1, 2, 4 and

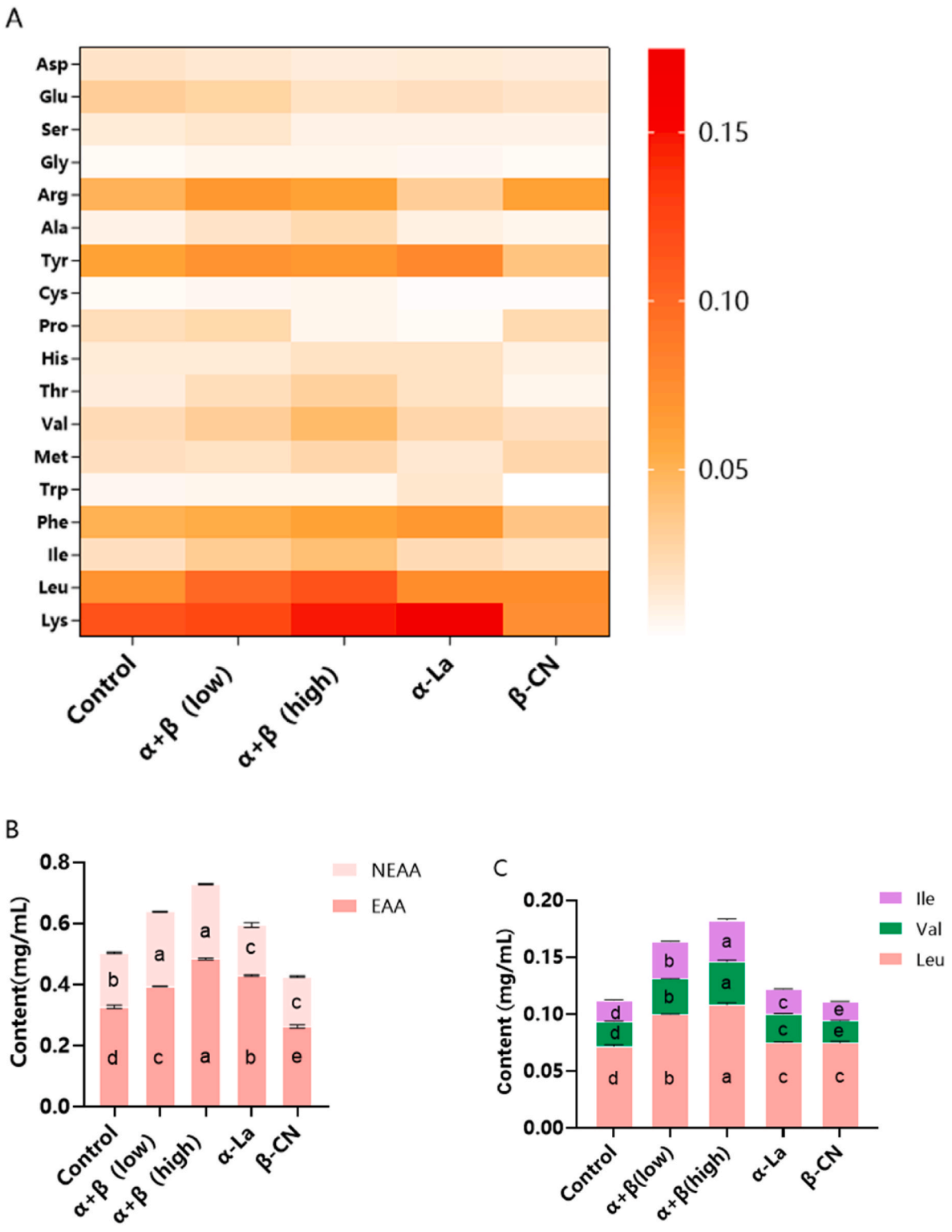

Fig. 3. Free amino acids of the digesta obtained from control, α+β (low), α+β (high), α-La, β-CN. (A) Free amino acids released after digestion. (B) Total amount of free amino acids. (C) Branched chain amino acid content. Values are mean ± standard deviation (n = 3) and values for samples with a different lowercase superscript differ significantly ($p < 0.05$).

10 μg/mL were prepared using Hanks equilibrium solution, and their absorbance was measured to create standard curves.

2.2.6. Determination of amino acids

The compositions and contents of the free amino acids were determined by HPLC (Agilent 1100, Agilent Technologies, Palo Alto, USA). The chromatographic column was Agilent Poroshell 120 HILIC-Z column (2.7 μm, 2.1 mm × 100 mm), and the mobile phase A was 20 mM ammonium formate and water. Mobile phase B: 20 mM ammonium formate and 90% acetonitrile water.

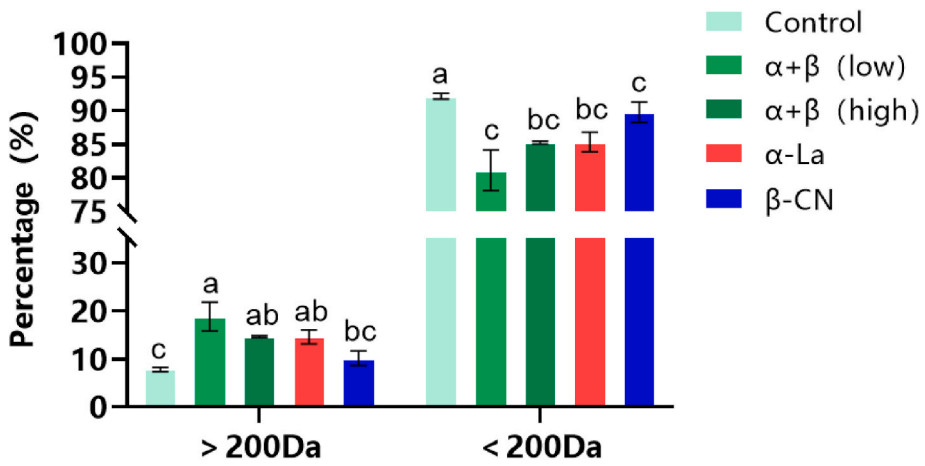

Fig. 4. Molecular weight distribution of polypeptide of cell transport products. α+β (low) with fortified low ratio of α-lactalbumin and β-casein; α+β (high) with fortified high ratio of α-lactalbumin and β-casein; α-La with pure α-lactalbumin protein and β-CN with pure β-casein protein. Values are mean ± standard deviation (n = 3) and values for samples with a different lowercase superscript differ significantly ($p < 0.05$).

2.2.7. RT-qPCR

The FastPure cell/tissue total RNA Isolation Kit V2 (Vazyme, Nanjing, China) was used to extract total RNA from Caco-2 cells. After the sample intervention, 500 μL of Buffer RL was added to lyse the cells. The resulting liquid was collected in a centrifuge tube and centrifuged. The supernatant was then taken and the cleavage product instructions provided in the kit were followed. The mRNA expression levels were measured using ChamQTM Universal SYBR qPCR Master Mix (Vazyme, Nanjing, China) and Roche LightCycler 96 (Roche, Basel, Switzerland) through RT-qPCR. Table .2 listed the prim sequence of target gene and β-actin.

2.2.8. Expression of transporter proteins

Following cell lysis, centrifugation was performed to remove cell debris. The protein concentration in the supernatant was measured using the BCA method. The transporter content was determined in accordance with the operation instructions of the ELISA kit.

2.2.9. Content of calcium, magnesium and zinc

Determination of Multi-elements in Food by GB 5009.268–2016 National Standard for Food Safety using ICP-MS (Thermo Fisher Scientific Inc, Waltham, Massachusetts, USA).

2.2.10. Molecular weight distribution of peptides

The digestive juice was centrifuged at 4 °C and 10000×*g* for 30 min. Filtered the resulting supernatant using a 0.45 μm membrane for later use. The determination was carried out using the Waters 1525 high-performance liquid chromatography (Waters, Milford, Massachusetts, USA).

2.2.11. Statistical analysis

All statistical analyses were performed using SPSS version 22.0 and GraphPad Prism 8. One-way ANOVA was applied and the statistically significant level was set at $p < 0.05$.

3. Results

3.1. Protein composition in different formulas

As shown in SDS-PAGE (Fig. 1), the band of α-CN in the control group was much darker and the band of α-La was lighter, that is, the content of α-CN in the control group was higher and the content of α-La was lower. With the addition of α+β, the band intensity of β-CN and α-La increased, especially for α+β (high)

3.2. Molecular weight distribution of polypeptide after digestion

At G0, the molecular weights of most groups were primarily distributed in the range of over 5 kDa (Fig. 2A and B). Specifically, β-CN was mainly distributed in the range of over 10 kDa. At I120, intestinal digestion was completed, and various digestive enzymes hydrolysed the protein, resulting in a molecular weight distribution primarily in the range of less than 0.5 kDa (over 70%). The degree of hydrolysis was similar among all groups. The molecular distribution of samples with minerals before and after digestion was similar to that of samples without minerals.

3.3. Free amino acids released after digestion

As can be seen from the Fig. 3, α-La was rich in lysine (Lys), leucine (Leu) and phenylalanine (Phe), and β-CN was rich in arginine. Compared to the control group, the samples showed a significant increase in the content of free amino acids and branched-chain amino acids after fortifying α-La and β-CN (Rafiee-Tari et al., 2019). The total content of α+β (high) of free amino acids and the content of branched-chain amino acids were higher than those of the control group by 31.74% and 62.97%, respectively. The order of essential amino acids in the mixed protein system was α+β (high) > α+β (low) > control. In the pure protein system, α-La > β-CN.

3.4. Molecular weight distribution of peptides transported to the basal side

The distribution of molecular weights for peptides and amino acids transported to the basal side indicated that 75% of the molecules were less than 200 Da, with the majority being at 130 and 150 Da (Fig. 4). This suggested that the absorbed nutrients consist mainly of small peptides and amino acids. There was little difference observed between each group.

3.5. Free amino acids transported by cells

Fig. 5 showed the content of a single amino acid on the basal side. The absorption rates of serine (Ser), proline (Pro), valine (Val), methionine (Met), isoleucine (Ile), Leu and Lys in α+β (high) were significantly improved (Fig. 5A). The total amount of amino acids transported to the basal side in the α+β (high) group was more than twice that of the control group and the content of branched-chain amino acids in α+β (high) was four times higher than the control (Fig. 5B). Moreover, 64.7% of the free amino acids transported to the basal side were positively correlated with their content in the digest, of which 6 amino acids were

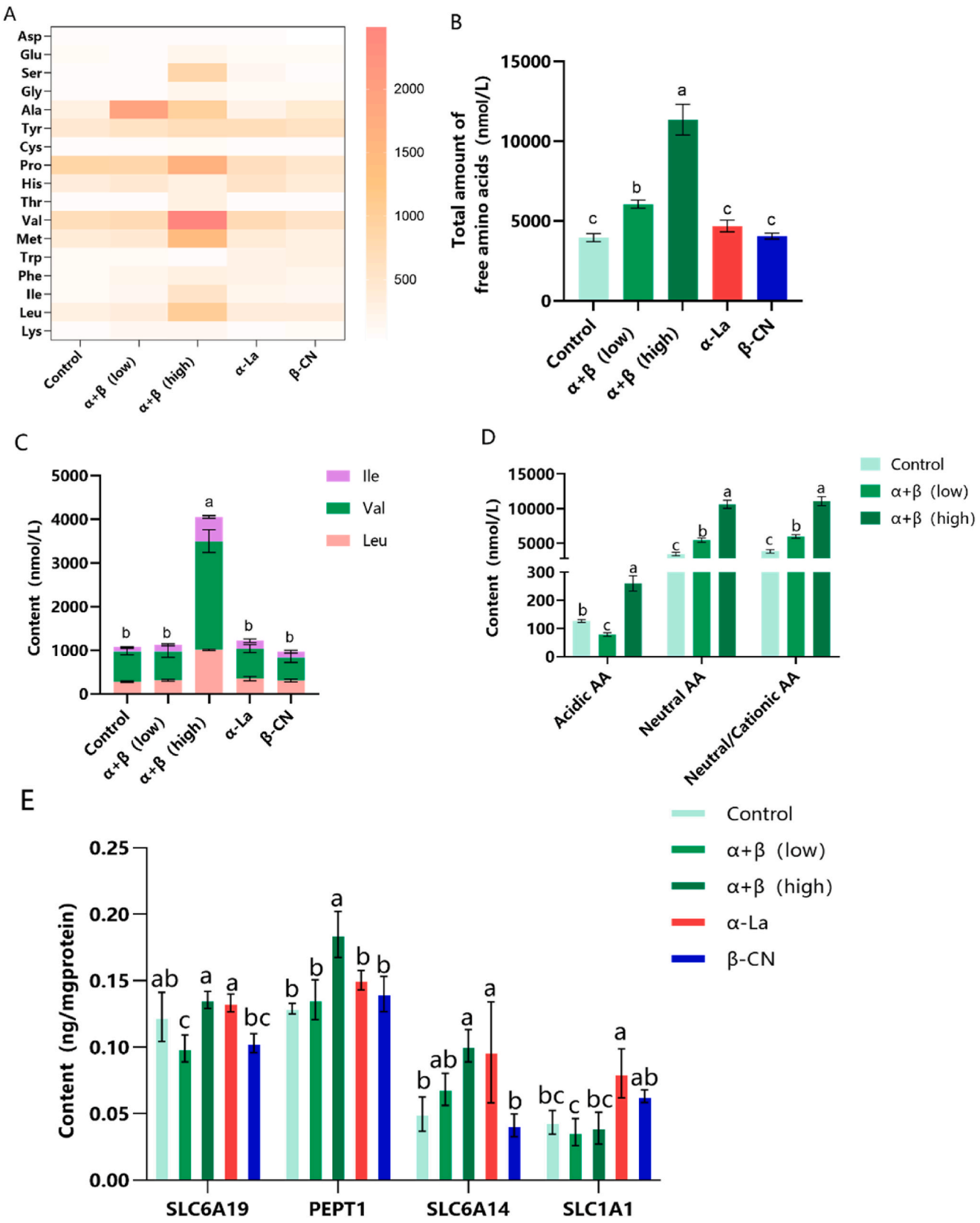

Fig. 5. Free amino acids transported by cells. α+β (low) with fortified low ratio of α-lactalbumin and β-casein; α+β (high) with fortified high ratio of α-lactalbumin and β-casein; α-La with pure α-lactalbumin protein and β-CN with pure β-casein protein. (A) Thermogram of single free amino acid content. (B) Total amount of transported free amino acids. (C) Based on the control group, the percentage of each amino acid increased. (D) Contents of acidic, neutral, neutral/cationic amino acids transported to the basal side. (E) Expression of amino acid and oligopeptide transporters. Values are mean ± standard deviation (n = 3) and values for samples with a different lowercase superscript differ significantly ($p < 0.05$).

Table 3
Correlation analysis of the free amino acid content in digestive juice and the free amino acid content transported to the basal side.

	Spearman Correlation coefficient
Asp	−0.544
Glu	−0.192
Ser	−0.644
Gly	0.917**
Ala	0.462
Tyr	0.5
Cys	0.833**
Pro	−0.617
His	−0.454
Thr	0.577
Val	0.561
Met	0.628
Trp	−0.494
Phe	0.8**
Ile	0.867**
Leu	0.787*
Lys	0.891**

Note.
a: * indicates significant correlation.
b: α+β (low) with fortified low ratio of α-lactalbumin and β-casein; α+β (high) with fortified high ratio of α-lactalbumin and β-casein; α-La with pure α-lactalbumin protein and β-CN with pure β-casein protein.

Table 4
Percentage of polar amino acids in total amino acids and absorptivity of total amino acids.

	Percentage of polar amino acids in total amino acids	Absorptivity of total amino acids %
Control	54.29	0.48
α+β (low)	49.46	0.56
α+β (high)	48.44	0.98

Note.
a: α+β (low) with fortified low ratio of α-lactalbumin and β-casein; α+β (high) with fortified high ratio of α-lactalbumin and β-casein; α-La with pure α-lactalbumin protein and β-CN with pure β-casein protein.
b: n = 3.

significantly correlated: Aspartic acid (ASP), glutamic acid (Glu), Ser, Pro, histidine (His) and tryptophan (Trp) (Table 3). The trend of increasing branched-chain amino acids content were similar to that of the total amino acids content (Fig. 5C). The study analysed the levels of acidic, neutral, and neutral/cationic amino acids. There were significant differences in the levels of these amino acids, except for the acidic amino acids of α+β (low), which were lower than those of the control group (Fig. 5D). Table 4 showed the percentage of polar amino acids in total amino acids and the cellular uptake rate of total amino acids in the mixed protein system, and the data showed that there was no obvious relationship between them. The expression of transporters was regulated by substrates (Fig. 5E). In different sample groups, the expression of the neutral/cationic amino acid transporter SLC6A14 and the oligopeptide transporter PEPT1 increased with the increase in α+β, in particular the expression of SLC6A14 in the α+β (high) group was 103% of that of the control group. The expression of SLC6A19 and SLC1A1 was lowest in the α+β (low) group, with little difference. In the pure protein system, the expression of each protein was lowest in the β-CN group.

3.6. Transport of calcium, magnesium and zinc

Minerals can be absorbed through multiple pathways. Fig. 6A displayed the mineral absorption rates for each group. The groups that consumed pure protein had a more positive effect on the absorption of Ca and Mg compared to the mixed protein groups. This effect was particularly evident for purified α-La, which had the most significant promoting effect. Among the mixed protein groups, α+β (high) had the most acceleration effect on mineral absorption, especially for Zn. Regarding mineral absorption-related transporters, the expression levels of mixed groups increased with the fortification level of α+β (Fig. 6B). The highest content in all the transporters was consistently observed in α+β (high), followed by α+β (low), both of which were higher than the control. In the pure protein system, the expression levels were in the order of α-La < β-CN.

Furthermore, we measured the levels of intestinal link proteins, as illustrated in Fig. 6C. The gene expression levels of Claudin-2, Zo-1, and Zo-2 decreased with increasing levels of α+β. Claudin-3 exhibited the lowest expression level in the α-La group.

4. Discussion

Differences in protein composition between infant formula and breast milk can affect the digestibility and absorption properties, potentially impacting infant growth and health (Tari et al., 2019). Our previous studies have shown that adjusting the levels of α-La and β-CN affected the digestibility of infant formula. The closer α-La and β-CN content of infant formula to breast milk, the more similar digestion pattern to breast milk (Huang et al., 2022). The objective of this study was to investigate the impact of fortification of α-La and β-CN on the absorption characteristics of infant formula and minerals. To achieve this, we conducted an *in-vitro* static infant digestion model to obtain digest with different levels of α-La and β-CN fortification. Subsequently, we used Caco-2 cell model to examine the absorption characteristics of amino acids, peptides, and minerals. The Caco-2 cell line is a standard screening tool for predicting nutrient absorption in the small intestine and studying transport mechanisms (Haddad et al., 2023). The differentiated Caco-2 cells resemble human small intestinal epithelium, with microvilli and enzyme systems related to the brush border epithelium of the small intestine (Li et al., 2023; Picariello et al., 2013).

Amino acids can be absorbed through facilitated diffusion or secondary active transport (Goodman, 2010). Our study found that an increase in α+β fortification led to an increase in the content of amino acids transported to the basal side. This could be due to the higher levels of free amino acids released by the α+β (low) and α+β (high) groups during digestion compared to the control group (see Fig. 3). Furthermore, most of the free amino acid contents in the digest were correlated with the absorbed free amino acids in the basal sides (Table 3). The difference in amino acid levels may affect transporter activity, as amino acid transporters can detect amino acid signals and respond accordingly (Hyde, Taylor, & Hundal, 2003). SLC6A19 is a neutral amino acid transporter, which is the main transporter of neutral amino acids in intestinal epithelial cells and acts as the main medium to deliver neutral amino acids to systemic circulation (Javed & Bröer, 2019). SLC6A14 is a unique amino acid transporter that transports all amino acids except glutamic acid and aspartic acid and has a broad substrate specificity (Karunakaran et al., 2011). The expression of transporters increased with the increase in α+β which, interestingly, in turn promoted the increase in substrate uptake. Furthermore, oligopeptides that are absorbed by cells are hydrolysed into amino acids. This is another significant method of amino acid absorption. Fortification of α+β may cause more oligopeptides to be absorbed into cells because α+β (high) can increase the release of peptide segments (Huang et al., 2022), thus increasing the amino acid content on the basal side. Among them, significant increases were observed in the transport of Leu, Ile, Val, tyrosine (Tyr), Pro, alanine (Ala), glycine (Gly), Ser and Glu to the basal side. The percentage of Ser transported to the basal side in the α+β (high) group (22.6%) was higher than in the control group (Fig. 5), which may be due to the release of amino acids in the body after absorption of the serine-containing peptide (Taub, Moss, Steffansen, & Frokjaer, 1998).

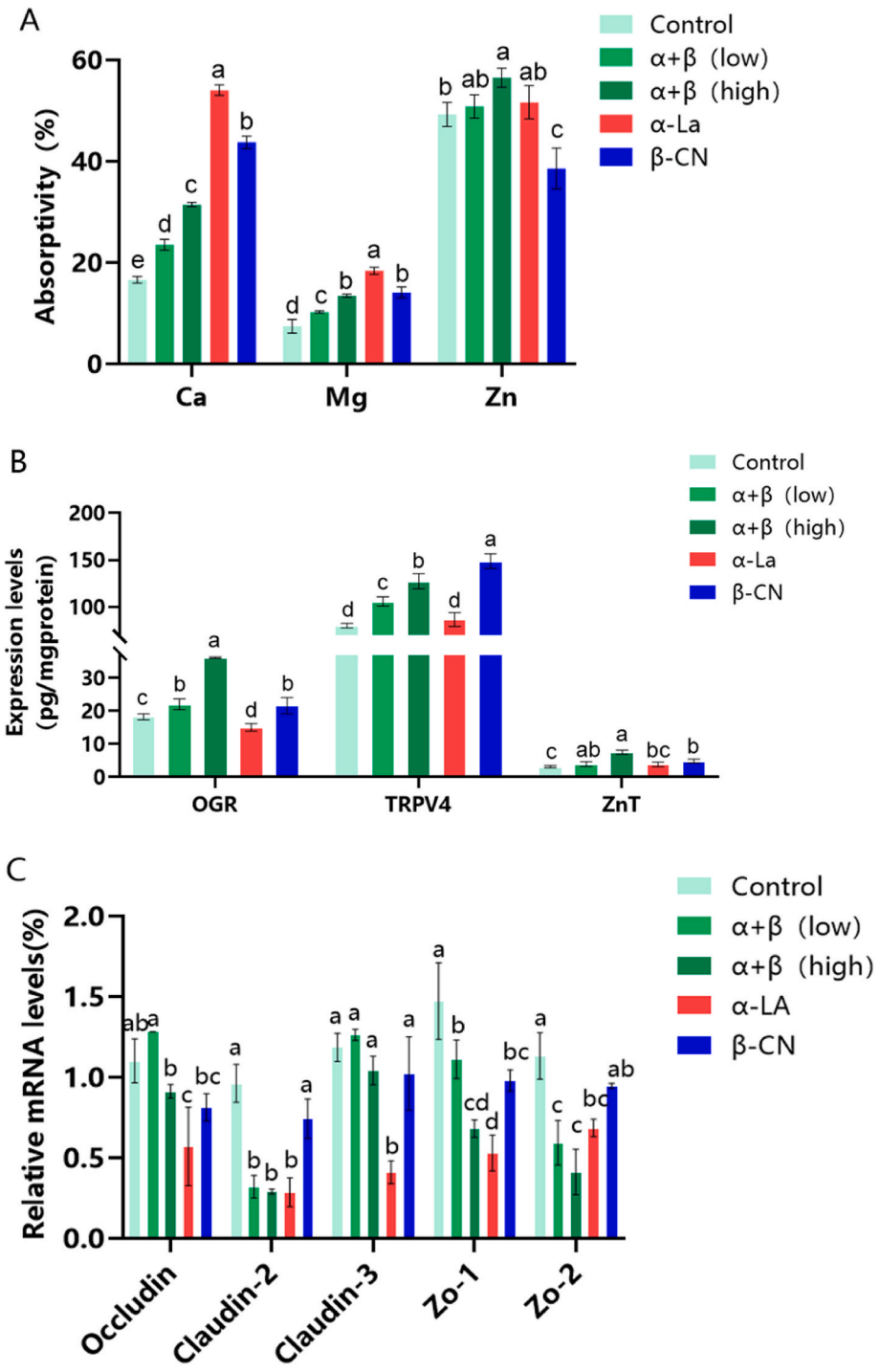

Fig. 6. The results of mineral transport by cells and the expression of mineral absorption-related proteins. α+β (low) with fortified low ratio of α-lactalbumin and β-casein; α+β (high) with fortified high ratio of α-lactalbumin and β-casein; α-La with pure α-lactalbumin protein and β-CN with pure β-casein protein. (A) Absorptivity of minerals. (B) Expression of mineral transporters. (C) Relative expression of intestinal connexin mRNA. Values are mean ± standard deviation (n = 3) and values for samples with a different lowercase superscript differ significantly ($p < 0.05$).

Ser can alleviate liver oxidative stress by regulating the expression of genes related to glutathione synthesis and increasing glutathione concentration (Zhou, He, et al., 2017; Zhou, He et al., 2018). The increased content of Ser transported into the body may help infants resist oxidative stress (Zhou, Zhang, Wu, Wan, & Yin, 2018), maintain mitochondrial function, and alleviate intestinal inflammation (Zhou, Zhang, et al., 2017). In addition, the content of branched-chain amino acids transported to the basal side also increased, and α+β (high) was about 200% of that of the control group (Fig. 5D). The increase in levels of branched-chain amino acids is important for infant growth and development as they play a crucial role in muscle synthesis and growth hormone secretion (Amari, Shahrook, Namba, Ota, & Mori, 2020; Suryawan, Rudar, Fiorotto, & Davis, 2020). Therefore, the infant formula fortified with α+β may have a positive impact on maintaining the health and promoting the growth of infants. However, the content of Ala, His, Trp and cysteine (Cys) transported into basal side were not increased with the increase of their contents in digest within 2 h, which were probably due to their low affinities. The main absorption mode of

amino acids and oligopeptides is active transporter (Kovalchuk & Nalecz, 2021). Transporter will preferentially transport the amino acids with higher affinities (Goulart et al., 2014; Sakai et al., 2020).

Besides protein absorption, we also studied the effects of different groups of samples on mineral absorption. With the increase of α+β fortification, the absorption rate of minerals and the expression of corresponding transporters also increased. Among these groups, α-La had the strongest promoting effect on the absorption of Ca and Mg (Fig. 6A). Protein promotes mineral absorption through a variety of mechanisms, including the use of peptides produced by digestion to promote mineral entry into cells. Peptides resulting from the digestion of α-La and β-CN have a high negative charge content (Vegarud, Langsrud, & Svenning, 2000). For instance, casein phosphopeptides can bind with divalent cations to create soluble complexes, which effectively enhance mineral absorption. In addition, the combination of minerals changes the spatial structure of peptide segments and some resist further digestion by gastrointestinal enzymes, allowing them to easily reach and be absorbed by intestinal epithelial cells. Therefore, increasing the levels of α-La and β-CN can improve the transport and absorption of minerals by cells. Besides, the protein-protein interface has a wide range of sizes and shapes, with shape complementarity usually described by core and peripheral regions, and a few hot-spot residues contributing most to binding affinity (Martin & Frezza, 2022). In the protein-protein complex, the side chain usually changes from one rotational isomer to another, usually involving 60 or more rotations. This structure may lead to stable mineral binding sites in the complex, thereby enhancing absorption, but our data cannot clearly explain the specific mechanism.

In addition to increasing the chances of entering the basal side by combining with protein, minerals can be absorbed directly through the intercellular space. The structure of the intestinal tight junction protein is highly dynamic, allowing cells to continually adapt to their environment. The tight junction is composed of various occludins, claudins and zonula occludens (ZOs), which link neighbouring cells to the actin cytoskeleton to form the epithelial barrier (Heinemann & Schuetz, 2019). Tight junction proteins can also regulate cell polarity and play an important role in the responsiveness, transport function and proliferation of directional cells. Over expression of different tight junction proteins in cell lines affected epithelial resistance and permeability to different ions, and these changes depend on the exact tight junction proteins expressed (Goff, 2018). Claudins can form paracellular ion channels with different specificities and permeabilities, which is the main determinant to regulate the paracellular permeability of tightly bound cells (Lynn, Peterson, & Koval, 2020). The expression level of Claudin-2 was control > α+β (low) > α+β (high), which means that the pore size was appropriately increased, promoting the flow of cations into cells and increasing the uptake of Ca.

Besides, cells also absorb minerals through ion channels. Permeability-sensitive transient receptor potential channel (TRPV4) is a type of non-selective cation channel that is widely distributed in organisms and permeable to Ca^{2+} and Mg^{2+} (Garcia-Elias et al., 2014; Nilius, Vriens, Prenen, Droogmans, & Voets, 2004). The regulation of its activity is regulated by Ca^{2+} and the combination of Ca^{2+} and calcium ion-binding protein-calmodulin (CaM) will enhance the activity of TRPV4, thereby promoting the influx of Ca^{2+} and Mg^{2+}. OGR1 (SLC41A1) is a Mg^{2+} transporter, a member A1 of solute carrier family 41, and is currently a well-studied Mg^{2+} transporter at present (Kolisek et al., 2008). ZnT1 is a specific transporter for transporting intracellular Zn^{2+} into the body (Nishito & Kambe, 2019). In the mixed protein system, the expression levels of all three transporters increased as the fortification ratio increased, and there were significant differences between the groups.

However, there were some limitations. The sample used in this study mainly contained protein and lactose and they were different from real infant formula, which also contained fat and vitamins. Our study didn't consider the influence of fat composition on the protein digestion. Furthermore, our study was carried out by *in-vitro* static digestion model combined with Caco-2 cell model, which may not be exactly the same as human gastric peristalsis and digestive juice discharge (Xin, Zhao, Tian, & Li, 2023). Therefore, the results may not be able to reflect the real digestion and absorption properties as that in clinical human study.

5. Conclusion

In sum, the total amount of amino acids and the content of essential amino acids in the digest increased in the group fortified with α-La and β-CN infant formula. Furthermore, the content of amino acids transported to the basal side of cell also increased with the increase of fortification level of α+β particularly in the α+β (high) group, which reached 11.343 μmol/L. In addition, α-La and β-CN promoted the absorption of Ca, Mg and Zn, which may have positive effect on the infant's own growth and development.

CRediT authorship contribution statement

Mengyu Han: Writing – original draft, Software, Methodology, Investigation, Formal analysis, Data curation. **Xuemin Wang:** Writing – review & editing, Resources, Methodology. **Kai Zhang:** Visualization, Methodology. **Ignatius Man-Yau Szeto:** Visualization, Methodology. **Sufang Duan:** Project administration, Methodology. **Yalu Yan:** Validation, Investigation. **Biao Liu:** Project administration. **Kasper Hettinga:** Writing – review & editing. **Lina Zhang:** Writing – review & editing, Validation, Project administration, Conceptualization. **Peng Zhou:** Writing – review & editing, Supervision, Project administration.

Declaration of competing interest

There is no interests or conflicts to be declared.

Data availability

Data will be made available on request.

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Abbreviations

α-lactalbumin (α-La)
β-casein (β-CN)
Aspartic acid (Asp)
Glutamic acid (Glu)
Serine (Ser)
Glycine (Gly)
Alanine (Ala)
Tyrosine (Tyr)
Cysteine (Cys)
Proline (Pro)
Histidine (His)
Threonine (Thr)
Valine (Val)
Methionine (Met)
Tryptophan (Trp)
Phenylalanine (Phe)
Isoleucine (Ile)
Leucine (Leu)
Lysine (Lys)

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fbio.2024.103948.

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