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Original Article

Antioxidant Potential, Nutrition, and Metabolomic Analysis of the Amino Acid Content of Infant Formula Milk for 7–12 Months and Predicted Benefits in Indonesia

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Abstract

The research aims to analyze the calcium (Ca), protein, and various amino acids contained in various samples of formula milk in circulation and given to babies as an alternative or additional nutrition. Samples of formula milk aged 7–12 months used in the research were of 4 types, coded A, B, C, and D. Calcium (Ca) content analysis was used using the Atomic Absorption Spectrophotometry (AAS) instrument; total protein was determined using the Kjeldahl method; and metabolomic analysis of amino acids were determined using a High-Performance Liquid Chromatography (HPLC) instrument. The calcium content of milk samples was 4.38 ± 0.03 mg/g (code A), 4.63 ± 0.03 mg/g (code B), 4.92 ± 0.08 mg/g (code C), and 5.48 ± 0.06 mg/g (code D); total protein content was 0.50 ± 0.01 mg/g (code A), 0.50 ± 0.03 mg/g (code B), 0.51 ± 0.01 mg/g (code C), and 0.75 ± 0.00 mg/g (code D); as well as the metabolomic of amino acid content contained in milk samples with code B (15 types), code D (13 types), and codes A and C have the same number of amino acid types (9 types).The research sample formula for milk contains important nutrients, including calcium, protein, and various metabolomic amino acids, which are important for the growth and development of babies aged 7–12 months.

Keywords: 7-12 months, AAS, amino acids, calcium, formula milk, HPLC.

Introduction

postnatal growth and development. Breast milk contains satisfactory macro- and micronutrients that babies need after birth (Ahern *et al.,* 2019; Sánchez *et al.,* 2021). Some of the nutritional contents contained in breast milk include protein, lactose, fat, calcium, phosphorus, and others (Ahern *et al.,* 2019; Gidrewicz & Fenton, 2014; Hanson *et al.,* 2016; Samuel *et al.,* 2020; Sánchez *et al.,* 2021). The development of knowledge about the composition of the nutritional content in breast milk has encouraged the health industry to produce substitutes (formula milk) that are close to the composition of breast milk, despite the fact that it cannot be denied that the content of breast milk is much more complete and better for the growth and development of babies (Sánchez *et al.,* 2021). Disruption of lactation production makes breastfeeding impossible due to conditions related to the modern era such as malnutrition, maternal absence, lack of lactation, food allergies, and other maternal health problems, so the existence of formula milk is very helpful and can be used as a substitute to meet the nutritional needs of growth and baby development (Gutiérrez, 2020).

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The milk formula is designed to have a composition that is almost identical to the composition of breast milk. When breast milk production is disrupted and does not produce milk, the best alternative to fulfill the baby's nutrition is by giving formula milk (Hageman *et al.,* 2019). When breastfeeding is not optimal and there is no milk substitute, it is estimated that it can cause nearly 600,000 child deaths every year, so formula milk is the best alternative as a recommended substitute for breast milk (Neves *et al.,* 2021; Rizvi *et al.,* 2022).

Apart from the protein and other nutrients contained in formula milk, antioxidant potential also plays a role in maintaining the body's strength and immune system. Antioxidants have the property of neutralizing the bad effects of free radicals. The components that provide antioxidant potential in milk are protein and various vitamins contained in milk, such as vitamins A and E (Stobiecka *et al.,* 2022). This study aims to identify the antioxidant potential and analyze the total protein content, various types of amino acids, and total calcium levels in various brands of formula milk in circulation. in Indonesia by taking samples of four types of formula milk aged 7–12 months in the city of Medan. The results of identifying antioxidant potential and analyzing various formula milks with various nutritional contents were then used to predict their nutritional benefits for babies. Protein is one of the macronutrients contained in milk, which has various essential amino acids and amino groups for the biosynthesis of non-essential amino acids and, if excessive, is used as an energy reserve. Calcium is an important mineral contained in milk that functions for the formation, development, and maintenance of bones (Masotti *et al.,* 2020)

Material and Methods

Materials

The equipment used includes glassware, analytical balance, vortex, Kjeldahl equipment, measuring glass, thermostat, AAS, UV-Vis spectrometer, and HPLC (Thermo Scientific Brand). Ingredients include lanthanum chloride heptahydrate ($LaCl_3.7H_2O$, Merck), distilled water, HNO_3 (Merck), 3% boric acid, HCl (Merck), NaOH (solid), DPPH (Merck), and ethanol (Merck). This research used four types of formula milk brands intended for babies aged 7–12 months circulating in Indonesia, which were obtained from supermarkets in the city of Medan. The four formula milks with different brands are coded A, B, C, and D (Supplementary).

Determination of total calcium contents

The determination of the total calcium content of the four types of formula milk brands was carried out using flame atomic absorption spectrophotometry. The four types of formula milk were dried in an oven at 105^0C until a constant weight was obtained. Then, the sample was carbonized and burned at 480^0C for 1 hour. After that, ashing was continued using a crucible in a muffle furnace at a temperature of 500^0C for 30 minutes, and a grayish-white color was obtained. The ash obtained was added to 10 mL of 0.1 M HNO_3 in a 50 mL volumetric flask and filled with deionized distilled water. Then 10% lanthanum chloride heptahydrate ($LaCl_3.7H_2O$, Merck) was added. Analysis was carried out with series flame atomic absorption spectrophotometry (tool type) equipped with a GLITE data station, a background correction system (deuterium lamp), and a calcium cathode lamp (Pietrzak-Fiećko & Kamelska-Sadowska, 2020).

Determination of total protein

The determination of total protein from each formula milk sample was carried out using the modified Kjeldahl method. Each formula milk sample was weighed at 5 g and put into a flask, followed by the addition of 0.5 g of copper sulfate, 0.7 g of potassium sulfate, and 20 mL of concentrated sulfuric acid. The sample is initially destroyed at a low temperature until it reaches normal hot conditions and continues to be destroyed at a heating temperature until a clear liquid is formed, then cooled. The resulting digestion solution was added to 250 mL of distilled water and then put into a distillation flask. The series of distillation tools is connected to a storage flask containing 15 mL of 3% boric acid, 70 mL of water, and 5 drops of indicator. Add 80 mL of sodium hydroxide solution to the distillation flask until the solution becomes very alkaline. Then the distillation process has been connected to the reservoir flask, and the distillation process continues until 150 mL of distillate is collected. The condenser flask was rinsed with distilled water. The Erlemeyer flask holding the distillate was neutralized with a portion of HCl acid (Rizvi *et al.,* 2022). The determination of total protein content is expressed by the nitrogen content contained in the sample and is calculated using the formula:

$$N = \frac{(V-B)\ x\ Ne\ x\ f\ x\ 14\ x\ 1000}{A}$$

where:
N: Nitrogen content (mg/g)
V: Volume of hydrochloric acid solution in titration (mL)
B: Volume of hydrochloric acid solution obtained in blank titration (mL)
Ne: normality expected volume of hydrochloric acid solution (0.02 or 0.05 N),
f: hydrochloric acid normality correction factor
A: sample weight (mg).

$$\%\ N = \frac{Mass\ of\ N\ in\ sample}{Massa\ of\ analyzed\ sample}\ x\ 100$$

Crude protein = % N x F
where:
F: correction factor for food samples whose value is generally 6.25 (da Silva *et al.,* 2016; Rizvi *et al.,* 2022).

Amino acid of metabolomic analysis with an HPLC instrument

The determination of amino acid of metabolomic content from each formula milk sample was carried out using a high-performance liquid chromatography (HPLC) instrument. Standard amino acid solutions were diluted using distilled water to produce concentrations of 0 ppm, 0.5 ppm, 1 ppm, and 2 ppm. Amino acid analysis was carried out using HPLC (Thermo Scientific Brand). A LiChrospher 100 RP-18 column (5 m) was used for the analysis of 10 µL of samples. Mobile phase A consisted of CH_3OH, phase B was 65% CH_3OH, and the reaction mixture contained 50 mM sodium acetate, tetrahydrofuran (THF), and a ratio of 2:96:2 at pH 6.8. Gradient separation with a constant moving flow rate of 1.5 mL/min. The detector uses a Thermo Dionex UltiMate 3000 RS Fluorescence Detector with an excitation wavelength of 300 nm and an emission wavelength of 500 nm (Slobodianiuk *et al.,* 2021; Xu *et al.,* 2020).

Identification of antioxidant potential

Determination of the antioxidant potential of milk samples using the DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) method. The protein extracted from each milk sample was made to a concentration of 1000 using ethanol solvent, then filtered using Whatman No. 1 filter paper. The filtrate was tamped and ready for further use. DPPH acted as a source of free radicals at a concentration of 0.4 mM DPPH. The short procedure carried out was taking 100 µL of each milk sample filtrate, then adding 1 mL of 0.4 mM DPPH, then adding ethanol to the 5 mL mark, and incubating for 30 minutes. After reaching the incubation time, the absorbance of the mixture was measured at 517 nm using UV-Vis spectrophotometry. The determination of antioxidant potential is expressed by the inhibition value obtained from using the formula (Sinaga *et al.,* 2022; Situmorang *et al.,* 2022; Tambunan *et al.,* 2024):

$$\text{Inhibition } \% = [\frac{A_{blank} - A_{sample}}{A_{balnk}}]\ x\ 100$$

Results

Calcium contents

The determination of calcium contents from each sample of formula milk for ages 7–12 months was carried out using the AAS instrument. The calcium contents of each formula milk sample are shown in Table 1.

Table 1. Total calcium content of each formula milk in the samples

No.	Milk code formula	Experiment contents		Packaging contents	
		(mg/g)	(%)	(mg/100g)	(%)
1.	Code A	4.38 ± 0.03	0.88 ± 0.01	530	0.53
2.	Code B	4.63 ± 0.03	0.93 ± 0.01	329	0.29
3.	Code C	4.92 ± 0.08	0.98 ± 0.**02**	448	4.48
4.	Code D	5.48 ± 0.06	1.10 ± 0.01	395	3.95

Note: levels are expressed as mean ± SD; measurements were carried out in triplicate.

Calcium (Ca) is one of the most abundant minerals in the body. The presence of calcium in the body is vital, especially for newborns and developing babies. The presence of calcium plays an important role in intracellular metabolism, bone growth, blood clotting, nerve conduction, muscle contraction, and heart function (Bass & Chan, 2006; Mehrotra *et al.,* 2019). The presence of calcium is very essential to determine in order to fulfill the intake needed by babies during the growth period from formula milk, which is used for babies as a nutritional intake other than breast milk. Calcium intake given to babies is the highest type of mineral, especially for babies born prematurely, in order to maintain long-term bone formation and health (Gates *et al.,* 2021; Mihatsch *et al.,*2021). The presence of calcium in formula milk intake is important for increasing bone mineralization and preventing osteopenia of prematurity, increasing blood vessel tone and myocardial function, supporting neural adaptation (Gates *et al.,*2021), as well as determining optimal cognitive development along with other minerals (iron and zinc) (Hamner *et al.,*2016). The calcium requirements for babies born prematurely far exceed the intake requirements for babies born normally (full term). Term babies with a birth weight of 3.5 kg need 57 mg/kg/day of calcium, while premature babies of 1 kg need 200 mg/kg/day (Gates *et al.,* 2021). Based on the results of the analysis of the presence of calcium levels in each formula milk consumed by babies aged 7–12 months, it is still in accordance with the baby's needs, and the highest calcium level is in the code D formula milk sample (5.48 ± 0.06 mg/g), and the lowest level among the four The sample was code A formula milk (4.38 ± 0.03 mg/g). The calcium content in the research sample determined using the AAS instrument compared to the calcium content written on the packaging showed a difference. However, the difference obtained was not significant. This is highly dependent on the homogeneity of calcium, sampling from a mixture of formula milk powder and preparation factors, and the instrument used.

Total protein content

The determination of the protein content of each formula milk sample for ages 7–12 months was carried out using the Kjeldahl method. The total protein content of each formula milk sample is shown in Table 2.

Table 2. Total protein content of each formula milk in the samples.

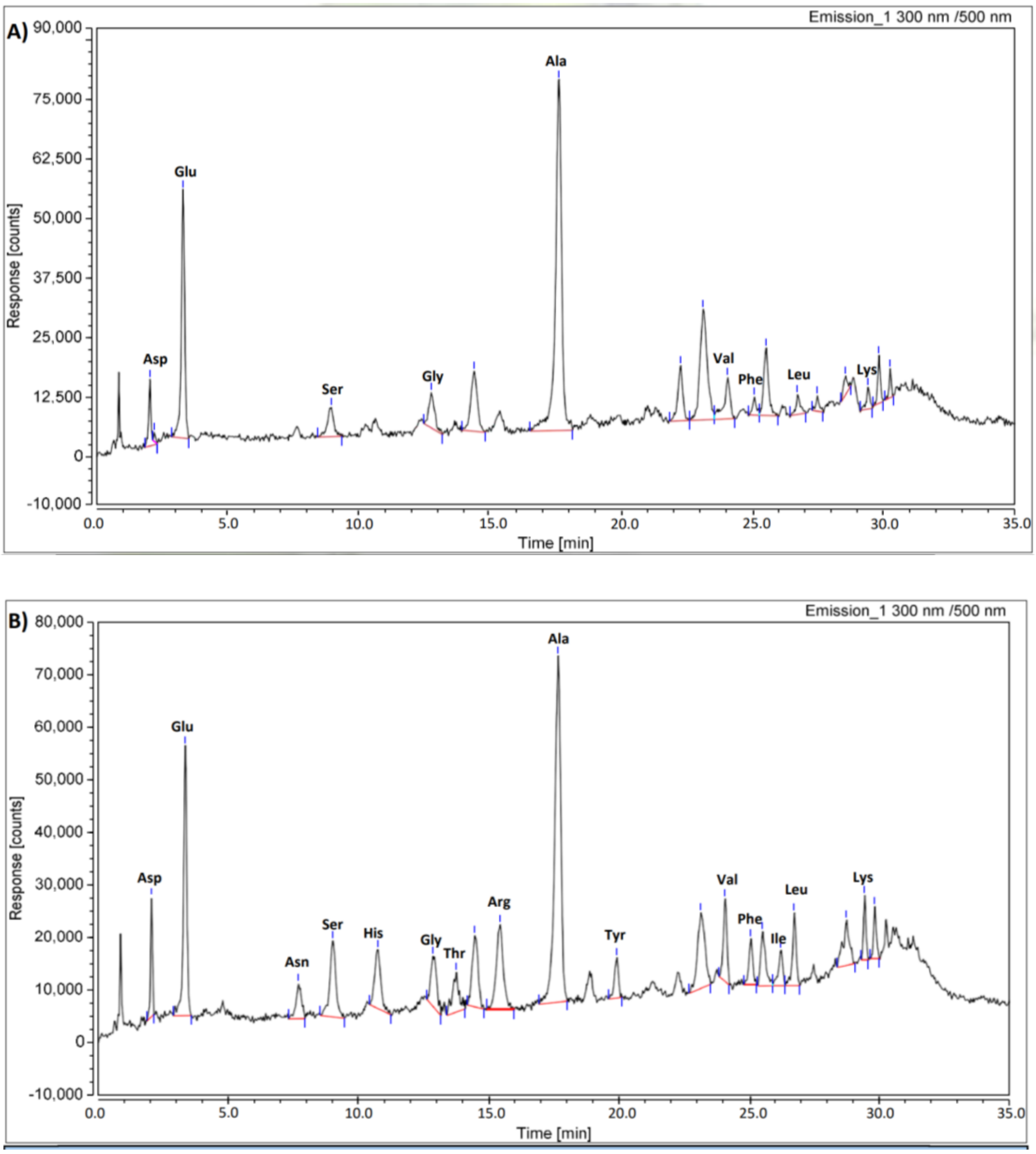
No.	Milk code formula	Experiment contents		Packaging contents	
		(mg/g)	(%)	(g/100g)	(%)
1.	Code A	4.38 ± 0.03	0.88 ± 0.01	10.00	10.00
2.	Code B	4.63 ± 0.03	0.93 ± 0.01	15.00	15.00
3.	Code C	4.92 ± 0.08	0.98 ± 0.02	10.00	10.00
4.	Code D	5.48 ± 0.06	1.10 ± 0.01	9.50	9.50

Note: levels are expressed as mean ± SD; measurements were carried out in triplicate.

Based on the results of the analysis of protein levels (Table 2), it was determined that the highest and lowest protein levels in formula milk were in codes D, C, B, and A. The determination of protein levels in formula milk samples was carried out using the Kjeldahl method, which is expressed by determining the levels of nitrogen atoms contained in the sample and stating the total protein content contained in the sample. This factor also makes the experimental protein content measurement data different from the protein content contained in the formula milk packaging. In addition, homogeneity factors, sample preparation, and accuracy also contribute to this difference. The amount and quality of protein contained in milk is an important prerequisite for normal baby growth, whereas if its presence is below normal, then metabolic needs can cause growth disorders, malnutrition, and damage to the brain and other organs. The consequences of low protein intake and low absorption in the baby's body are a serious problem, especially in the case of babies born prematurely (Koletzko *et al.,* 2019; Purkiewicz *et al.,* 2023).

Analysis metabolomic of amino acid

The determination of the metabolomic of amino acids was carried out using an HPLC instrument due to the efficiency of the column, sensitivity, and selectivity of the detection system for the samples analyzed. The results of the analysis of the metabolomic of amino acids contained in each milk sample are presented in Figure 1 and Table 3. Each package of formula milk used in this research sample does not contain detailed information about the type and level of each amino acid. The packaging only provides information about the total weight of the amino acids contained. The information provided on the packaging regarding the quality of the total weight of amino acids does not guarantee the various types of amino acids contained in the formula milk. Various types of amino acids are very important for the growth and development of infants and are used as supplements or as the main substitute for breast milk.



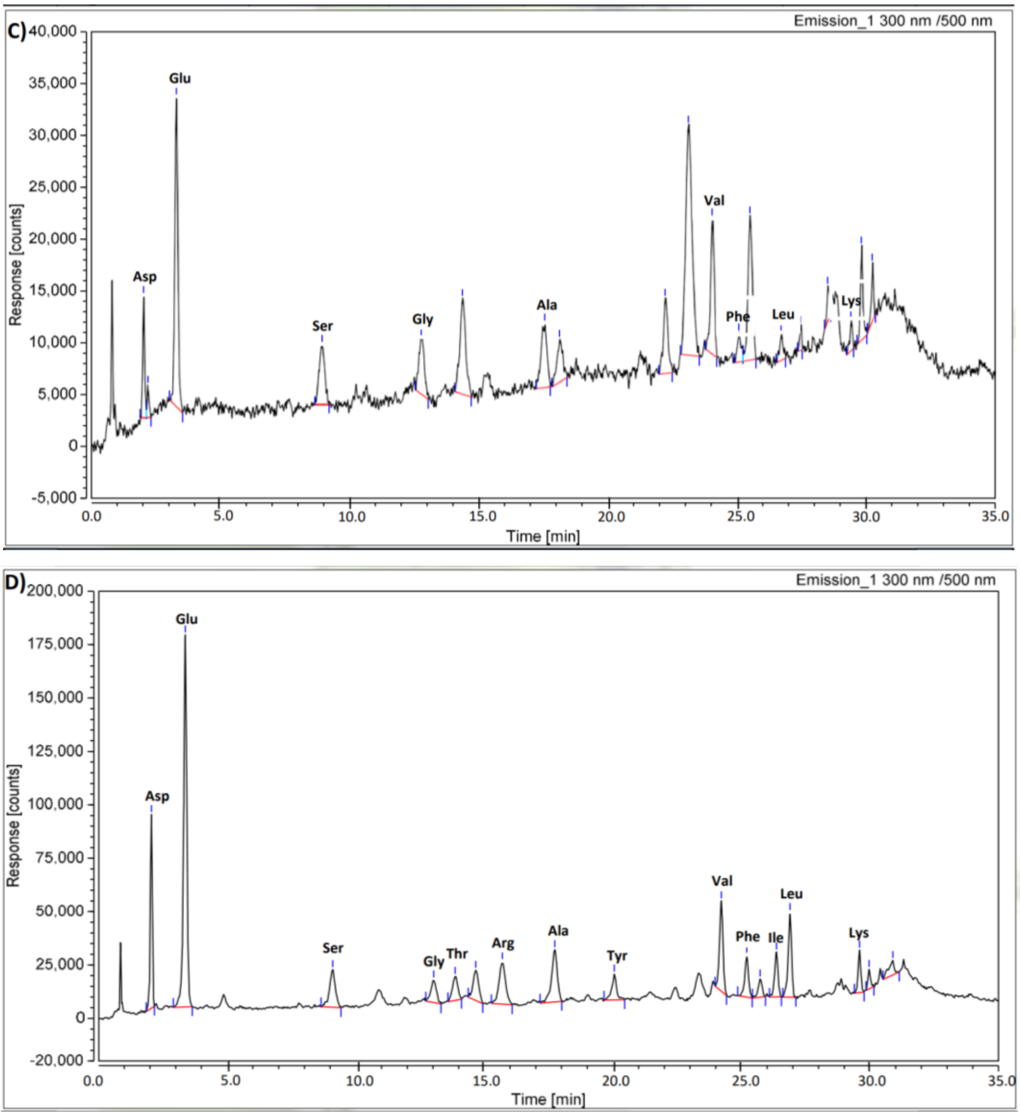


Figure 1. Metabolomic analysis of amino acid chromatograms from various milk samples using HPLC; A) code A, B) code B, C) code C, and D) code D.

Table 3. Results metabolomic analysis of amino acids from various milk samples using HPLC instruments

No.	Types of Amino Acids	Sample			
		Code A	Code B	Code C	Code D
1.	Aspartic acid (Asp)	√	√	√	√
2.	Glutamic acid (Glu)	√	√	√	√
3.	Asparagine (Asn)	-	√	-	-
4.	Serine (Ser)	√	√	√	√
5.	Histidine (His)	-	√	-	-
6.	Gysine (Gly)	√	√	√	√
7.	Threonine (Thr)	-	√	-	√
8.	Arginine (Arg)	-	√	-	√
9.	Alanine (Ala)	√	√	√	√
10.	Tyrosine (Tyr)	-	√	-	√
11.	Valine (Val)	√	√	√	√
12.	Phenylalanine (Phe)	√	√	√	√
13.	Isoleucine (Ile)	-	√	-	√
14.	Leucine (Leu)	√	√	√	√
15.	Lysine (Lys)	√	√	√	√

Based on Figure 1 and Table 3, the order of the most abundant-least metabolomic analysis of amino acids is that milk samples with code B (15 types), code D (13 types), and codes A and C have the same number of amino acid (9 types). The abundance of varied amino acids provides complete nutrition and good health benefits. Amino acids in the body have an important role in signaling, regulating gene expression, protein phosphorylation cascades, the main precursors in hormone synthesis, and biological importance (metabolism). The presence of various amino acids contained in milk will be useful as nutrients that correct problems, regulate cycles in the body better, and optimize the efficiency of metabolic transformation for baby growth and development (Li *et al.,* 2019; Wu, 2009). The levels of each amino acid were determined from the standard amino acid standard curve, and a linear regression equation was obtained (Table 4). A comparison of the various amino acid contents of each milk sample is presented in Figure 2.

Table 4. Metabolomic analysis of amino acid content from various milk samples

No.	Metabolomics of Amino Acids	Linear regression equation	R^2	Sample amino acid content (ppm)			
				Code A	Code B	Code C	Code D
1.	Aspartic acid (Asp)	y = 33638x + 641.02	0.9994	0.02	0.04	0.01	0.22
2.	Glutamic acid (Glu)	y = 35804x + 113.52	0.9999	0.21	0.21	0.11	0.71
3.	Asparagine (Asn)	y = 28497x + 115.25	0.9999	-	0.05	-	-
4.	Serine (Ser)	y = 42322x + 26.8017	0.999	0.04	0.09	0.03	0.11
5.	Histidine (His)	y = 14229x + 400.69	0.9991	-	0.19	-	-
6.	Gysine (Gly)	y = 54014x + 334.98	0.9991	0.04	0.05	0.03	0.06
7.	Threonine (Thr)	y = 40487x + 213.11	0.9999	-	0.06	-	0.07
8.	Arginine (Arg)	y = 421865x + 31.164	0.9999	-	0.23	-	0.28
9.	Alanine (Ala)	y = 45287x + 474.75	0.9997	0.44	0.39	0.03	0.13
10.	Tyrosine (Tyr)	y = 7437.4x + 106.38	0.9986	-	0.17	-	0.32
11.	Valine (Val)	y = 38997x + 176.71	0.9999	0.05	0.06	0.06	0.17
12.	Phenylalanine (Phe)	y = 20370x + 149.37	0.9999	0.03	0.06	0.02	0.15
13.	Isoleucine (Ile)	y = 34212x + 395.6	0.9998	-	0.03	-	0.08
14.	Leucine (Leu)	y = 28191x + 232.34	0.9999	0.02	0.07	0.01	0.21
15.	Lysine (Lys)	y = 4255.9x + 24.504	0.9999	0.17	0.32	0.09	0.57

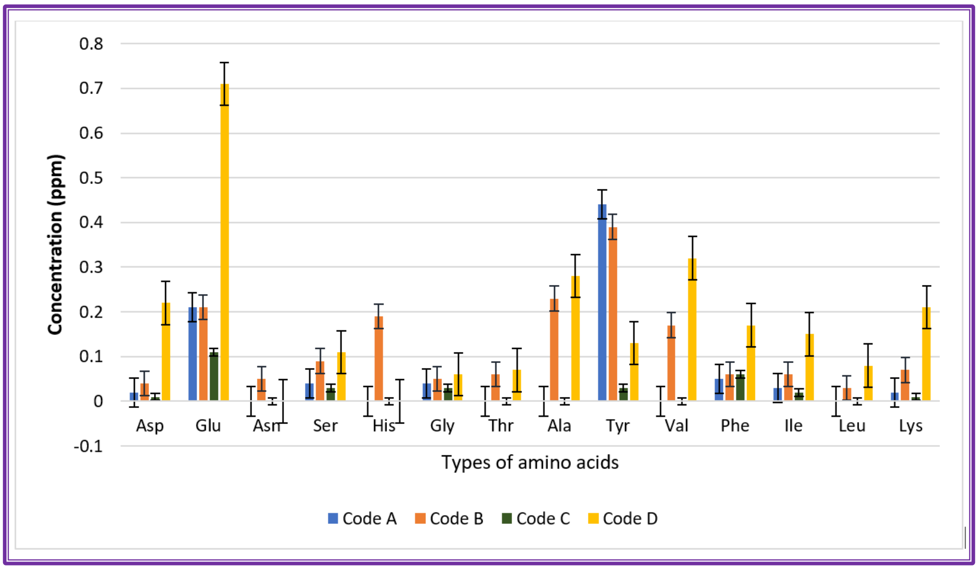


Figure 2. Comparison of various metabolomic of amino acid content of each milk sample.

Amino acids based on the body's ability to synthesize them are divided into two groups, namely essential and non-essential amino acids. Milk samples contain essential amino acids, including Histidine (His), Threonine (Thr), Valine (Val), Isoleucine (Ile), Arginine (Arg), Phenylalanine (Phe), Lysine (Lys), and Leucine (Leu), while the types of non-essential amino acids found in milk samples are Aspartic acid (Asp), Glutamic acid (Glu), Asparagine (Asn), Serine (Ser), Glycine (Gly), Alanine (Ala), and Tyrosine (Tyr) (Wu, 2009). The presence of various amino acids contained in milk samples will maintain the baby's growth and development, as well as keep the baby's metabolic system and body health in normal and good condition. Apart from that, amino acids also play an important role in the rapid growth of babies, maintaining the food digestive system and good absorption of nutrients, and protecting the baby's body from pathogens, viruses, bacteria, and fungi (Purkiewicz *et al.,* 2023).

Antioxidant potential of milk samples

Milk has a variety of additional nutrients that are formulated into a nutrient preparation that is very good for the body. In this case, formula milk is for babies aged 7–12 months. Many studies have shown that the proteins contained in milk provide antioxidant potential, and the quality of the antioxidants is greatly affected by the temperature and treatment time in which the milk is served (G. Niero *et al.,* 2017; Stobiecka *et al.,* 2022). The antioxidant potential of various milk samples is presented in Table 5.

Table 5. Antioxidant potential of various formula milk samples for ages 7–12 months.

No.	Milk formula (Concentration: 1000 ppm)	Inhibition (%)
1.	Code A	26.300 ± 0.001
2.	Code B	35.168 ± 0.001
3.	Code C	22.324 ± 0.001
4.	Code D	34.251 ± 0.001

Note: Measurements are expressed as mean + SD, with three repetitions

The measurement results in Table 5 show that each formula milk provides antioxidant potential in the range of 22.324–35.168%, with a standard deviation of 0.001 for each. The antioxidant potential of the highest-llowest formula milk samples starts with codes B, A, D, and C. The antioxidant potential provided by each milk sample collaborates positively with the data from the analysis of the content of various types of amino acids contained in the formula milk samples. The antioxidant potential provided actually really depends on the oxidative reaction of the protein resulting in a hydrolysis process and the release of amino acids, high temperature conditions during processing or brewing, and the conditions and duration of the milk preparation being contaminated in an open environment (Giovanni Niero *et al.,* 2019).

Conclusion

Formula milk is an appropriate substitute for babies when the productivity and system of the mother's mammary glands are disrupted in producing milk. Formula milk is designed to have a nutritional composition that is close to that of breast milk. This nutritional content is very important to maintain health and ensure that the baby's growth and development remain good and normal. One of the important nutritional contents found in milk is calcium and protein. Calcium plays a role in bone and protein formation with various amino acid contents, which play an important role in stimulating growth and development, maintaining the metabolic system, and warding off free radicals in the body because it has antioxidant properties. Various milk samples analyzed showed the content of calcium, protein, and various types of amino acids, both essential and non-essential, and showed antioxidant potential. In general, the order of nutritional content of each milk sample analyzed more completely is the formula milk sample aged 7–12 months with codes B, D, A, and C.

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Conflict of Interest

The authors declare no conflict of interest.

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