Harnessing the Power of a Novel Triple Chelated Complex in Fermented Probiotic Dairy Products: A Promising Solution for Combating Iron Deficiency Anemia

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Abstract

This study discovered and examined novel triple chelate complexes involving iron, ascorbic acid, and essential amino acids (AsA-Fe-AmA triple chelate complexes) for the first time. The mechanism of complexes formation was studied using FTIR spectroscopy and quantum chemical modeling. The produced complexes were shown to be suitable for fortifying food items with a pH of 3-7 that have not been exposed to heat treatment at temperatures over 75 °C for more than 15 minutes.

Thus, it was set that concentration for milk fortification should be 0.005 mol/L or less. In vivo experiments in rats models revealed that synthesized complex increases serum iron levels after a single application to reference values within 24 hours after oral administration. The iron level increased by 14.0 mmol/L at 2 mL of the complex. This fact makes it possible to consider the developed complex and developed fermented dairy product for the prevention of iron deficiency and iron deficiency anemia.

Research on the physicochemical and organoleptic qualities of milk enhanced with the discovered compounds was conducted. Furthermore, iron ascorbate threoninate, iron ascorbate methioninate, iron ascorbate lysinate, and iron ascorbate tryptophanate all had a beneficial effect on Lacticaseibacillus rhamnosus at concentrations as low as 0.0005 mol/L, which is significant for milk fermentation. A study of fermented milk products revealed that the most effective AsA-Fe-AmA triple chelate complex is iron ascorbate lysinate, which might be further investigated as a viable molecule for dietary fortification in iron deficient anemia. It was found that fortified fermented milk product has a titratable acidity of 67 ± 1 °T, pH = 4.38 ± 0.05 and viscosity of 2018 ± 142 Pa·s.

Key words: quantum chemical modelling; vitamin C; fortification; titratable acidity; sensory evaluation; dispersed composition; trace element; fermented probiotic dairy products.

Introduction

One of the problems of human health is the lack of macro- and microelements
in the diet. More than two billion people worldwide have low levels of iron, zinc, iodine, vitamin D and folic acid. Considering that macro- and microelements are necessary for most biological processes in the human body, the lack of these substances can have a significant harmful effect on the physical and mental health of children and adults, and the development of a number of alimentary-dependent diseases (goiter, anemia, rachitis, Beriberi disease, Bitot spots etc.) can be provoked.

Nowadays, iron deficiency (ID) stands as the most prevalent condition worldwide. ID negatively impacts on mitochondria. Fang et al. demonstrated its particularly detrimental effect on cardiac function, exacerbating heart failure. Neidlein et al. noted that ID reduces working capacity and muscular strength. According to Pasricha et al., ID can disrupt immunological and endocrine functioning, resulting in the development of iron deficiency anemia (IDA).

Mirza et al. reported that iron deficiency accounts for 50-80% of cases of iron deficiency. IDA is one of the top five causes of disability in women in 35 countries, accounting for many years of impairment. According to Garnder et al., the global prevalence of IDA across all ages was 24.3% in 2021, accounting for 1.92 billion prevalent cases, compared to 28.2% and 1.50 billion prevalent cases in 1990. The authors discovered significant disparities in the frequency of IDA across age, gender, and geography.

According to WHO, IDA affects 53% of children in Africa, 40% in Asia, and 26% in Europe. These figures reported higher than those reported by Stevens et al. in a pooled study of population-representative data spanning from 2000 to 2019. Consequently, the global dynamics of IDA observation are escalating annually. In response, the World Health Assembly Resolution endorsed a Comprehensive implementation plan on maternal, infant, and early child nutrition. This plan delineated a series of nutritional objectives aimed at having the incidence of IDA among women and children twice by 2025.

The major strategy for addressing ID and IDA involves supplementing the human diet with bioavailable forms of iron (iron gluconate, iron dextran complex,
Various substances are utilized to enhance the bioavailability of iron. For example, nitrogen alkaloids (piperidine) combined with vitamins and minerals are employed in the production of iron supplements. Iron-saccharide compounds shown excellent efficacy in IDA therapy in model trials with rats. However, current research indicates that taking iron complexes orally might have serious side effects.

Thus, Tolkien et al. conducted a meta-analysis of 43 studies including 6831 adult participants, revealing a significant association between iron sulfate supplementation and gastrointestinal-specific adverse effects. These effects predominantly contain gastrointestinal discomfort such as nausea and vomiting, constipation or diarrhea, flatulence, metallic taste, tooth discoloration, or epigastric pain. As Malesza et al. suggested, iron supplementation can trigger the formation of reactive oxygen species in the gut lumen and enterocytes, leading to an inflammatory process that affects the intestinal wall integrity. The increase in intestinal wall permeability leads to a complex of unfavorable variables. Excess unabsorbed iron from oral supplementation traverses the colon, where it serves as a critical growth factor for a wide range of pathogenic bacteria, fungi, and protozoa, as well as all neoplastic cells, affecting the gut microbiota considerably. Moreover, Moksnas et al. in a genome-wide meta-analysis involving publicly summary statistics covering 257,953 people identified 123 genetic loci associated with iron traits. These loci may be influenced by elevated serum iron and transferrin saturation levels commonly observed with iron supplementation.

Iron absorption presents one of the multifactorial adverse effects of oral iron supplementation as documented in literature. Therefore, the approach to developing bioavailable iron supplements should prioritize innovative methods that optimize iron absorption in order to improve IDA treatment efficacy and reduce the risk of adverse effects. Ascorbic acid (AsA), citrate, and amino acids (AmA) all help in iron absorption.

Ascorbic acid (AsA) enhances iron absorption by converting Fe (III) to Fe (II), facilitating its uptake. Noubactep et al. proposed a method to extract iron
from granular metallic iron (Fe\textsuperscript{0}) with AsA, forming a stable and bioavailable Fe(II) - AA complex used for drinking water fortification.

Additionally, Cian et al.\textsuperscript{52} created microcapsules containing iron and ascorbic acid utilizing chelating polypeptides derived from brewers' waste grain protein. These microcapsules provided high iron bioaccessibility with minimal contact with the food matrix, avoiding redox reactions or adverse effects. Although triple complexes have just recently been explored, they hold significant scientific and practical for producing stable and bioavailable iron formulations.

The aim of this study was to produce and investigate a triple chelated complex of the important trace element iron, ascorbic acid, and essential amino acids (AsA-Fe-AmA) for the development of functional dairy products, particularly milk and fermented dairy items. Milk and dairy products (fermented milk products) offer significant potential for fortification with essential trace elements due to their versatile composition, encompassing both fat and non-fat fractions, enabling the addition of both fat-soluble and water-soluble components. Resulting fortified fermented milk products can be used to prevent ID and its consequences, in particular, ID anemia.

To the best of our knowledge, these complexes were synthesized for the first time in this study, demonstrating considerable potential for both scientific and practical applications.

2. EXPERIMENTAL DESCRIPTION

2.1. Chemicals and materials

The study utilized reagent-grade chemicals and Grade A glassware. Distilled water with a conductivity of less than 1 μS/cm was used. Dia-M (Moscow, Russia) supplied essential amino acids such as L-Valine, L-Leucine, L-Isoleucine, L-Threonine, L-Phenylalanine, L-Tryptophan, L-Methionine, and L-Lysine monohydrochloride. The following chemicals were also purchased for the experiment: ascorbic acid (NeoFroxx, Einhausen, Germany), iron (II) sulfate (Lenreactive, St. Petersburg, Russia), barium hydroxide (Dia-M, Moscow, Russia), potassium persulfate, sulfosalicylic acid (Lenreactive, St. Petersburg, Russia), and
2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (Alfa Aesar, Ward Hill, MA USA).

The food matrix used was pasteurized 3.2% fat milk obtained from a local dairy plant (Stavropol Dairy Plant, Stavropol, Russia). *Lacticaseibacillus rhamnosus* and *Lactobacillus acidophilus* strains (Christian Hansen, Hoersholm, Denmark) were employed for fermenting milk products. Stavrechem (Stavropol, Russia) supplied agar-agar feeding medium for microbiological examination.

### 2.2. Synthesis of the AsA-Fe-AmA triple chelate complexes

The synthesis of AsA-Fe-AmA triple chelate complexes was carried out using our earlier approach, with some minor modifications. In summary, 0.0155 mol of the essential amino acid (L-valine, L-leucine, L-isoleucine, L-threonine, L-phenylalanine, L-tryptophan, L-methionine, L-lysine monohydrochloride) was combined with 0.0155 mol of ascorbic acid. The mixture was then treated with 0.0155 mol of barium hydroxide, 30 mL of distilled water, and 0.0155 mol of iron (II) sulfate. The barium sulfate was extracted from the resultant solution by centrifugation at 3000 rpm using a MicroCL 17R centrifuge (Thermo FS, Waltham, MA, USA). Figure 1 shows the synthesis strategy.
Figure 1. Scheme of synthesis of triple chelate complexes of the essential trace element iron with ascorbic acid and essential amino acids

2.3. Characterization of the AsA-Fe-AmA triple chelate complexes

The optical properties were studied using an SF-56 spectrophotometer (OKB "Spectrum", St. Petersburg, Russia). For the study, samples of the AsA-Fe-AmA triple chelate complexes were diluted 100 times with distilled water. The study parameters were as follows: slit width – 6 nm, accumulation time – 0.3 s, wavelength range – 400-1000 nm.

To investigate functional groups in the obtained materials, FTIR spectroscopy was performed. IR spectra were acquired on an FSM-1201 IR spectrometer with Fourier transform (Infraspek, Saint Petersburg, Russia) across a measuring range of 500-4000 cm\(^{-1}\).

Quantum chemical modeling of the AsA-Fe-AmA triple chelate complexes was performed using QChem software and the IQmol molecular editor (QChem, Pleasanton, CA, USA) using the following parameters: Calculation - Energy, technique - HF, basis: 6-31G, convergence: 5, force field: Chemical.

2.4. Stability of the AsA-Fe-AmA triple chelate complexes

A multifactorial experiment was carried out to explore the stability of the AsA-Fe-AmA triple chelate complexes under various technical parameter values. The input parameters included the medium's active acidity (pH), mixing time (τ, min), and solution temperature (t, °C). The output parameters were variations in optical density (Δ D). The optical density value was obtained using an SF-56 spectrophotometer (OKB "Spectrum", St. Petersburg, Russia). Table 1 shows the matrix from the multifactorial experiment.

Table 1. Matrix of a multifactorial experiment

<table>
<thead>
<tr>
<th>№</th>
<th>pH</th>
<th>t, °C</th>
<th>τ, min</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3</td>
<td>25</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>60</td>
<td>15</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>95</td>
<td>25</td>
</tr>
</tbody>
</table>
2.5 Study of the effect of the AsA-Fe-AmA triple chelate complexes on the dispersed composition of milk

Samples of the AsA-Fe-AmA triple chelate complexes were diluted by 10 (concentration 0.05 mol/L), 100 (concentration 0.005 mol/L), 1000 (concentration 0.0005 mol/L) and 10,000 (concentration 0.00005 mol/L) times with pasteurized milk.

The average hydrodynamic radius of casein micelles in milk was determined using the dynamic light scattering method employing the Photocor-Complex device (Photocor, Moscow, Russia), as described in our earlier work. The ζ-potential and electrical conductivity were measured using an acoustic and electroacoustic spectrometer DT-1202 (Dispersion Technologies, Bedford Hills, NY, USA). Titrated acidity was calculated using the technique reported by An et al. The pH was determined using an Expert 001 pH meter-ionomer (Econix-Expert, Moscow, Russia) paired with a combination silver-chloride electrode (EVL-1M3.1).

2.6. Investigation of the antioxidant activity of milk products with the AsA-Fe-AmA triple chelate complexes

The antioxidant activity was assessed using the technique reported by Piskov et al., with slight adjustments. In brief, 5 mL of a 7 mM solution of 2,2-azino bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 1 mL of 14.7 mM potassium persulfate were combined and stored at room temperature in the dark for 24 hours.
Prior to analysis, the ABTS solution was diluted with distilled water to achieve an optical density of 0.70 (± 0.02) at λ = 734 nm. Subsequently, 0.25 m/L of 0.5 mol/L AsA-Fe-AmA triple chelate complexes solution was added to 100 mL of milk. Then, 1 mL of sample and 1 mL of sulfosalicylic acid were centrifuged at 13,000 rpm for 5 minutes in a MicroCL 17R centrifuge (Thermo FS, Waltham, MA, USA).

The sample was then divided into 0.02 mL aliquots and to each aliquot, 1.98 mL of the ABTS solution was added. An SF-56 spectrophotometer (OKB "Spectrum"; St. Petersburg, Russia) was used to detect absorption at 734 nm after 3 minutes of mixing. A 1 mM trolox solution was employed as a standard. The antioxidant activity was reported in mg of trolox equivalents per one milliliter of the sample (mg TE/mL).

2.7. Sensory analysis

For sensory study, 10 experimental batches of dairy and fermented milk products were prepared, each comprising 5 samples. Iron (II) sulfate, an inorganic version of the crucial trace element iron, served as the control sample. Following the requirements of ISO 22935-2:2023, a committee of 10 untrained panelists was assembled to evaluate the sensory attributes of the samples using a 5-point scale.

2.8 Qualitative microbiological assessment

*Lacticaseibacillus rhamnosus* cultured on agar-agar medium was qualitatively assessed following the method described by Alonso-Roman et al. with minor modifications. To prepare the microbial suspension, the culture was rinsed off the agar with sterile distilled water. The suspension was then filtered through a sterile cotton-gauze filter and diluted with sterile distilled water to a concentration equivalent to 1.0 unit per the McFarland standard, which corresponds to 300 × 106 CFU/mL.

For research purposes, solutions of the AsA-Fe-AmA triple chelate complexes were produced at concentrations of 0.05 mol/L, 0.005 mol/L, 0.0005 mol/L, and 0.00005 mol/L. *Lacticaseibacillus rhamnosus* suspensions (100 µL) were inoculated onto test tubes containing the AsA-Fe-AmA triple chelate complexes solutions.
Then they were plated onto Petri dishes with agar-agar, and incubated in a thermostat (Binder GmbH, Tuttlingen, Germany) at 37°C for 24 hours. The growing colonies were counted without opening the Petri plates and by flipping them upside down. Each tallied colony was marked with a dot with a fountain pen.

2.9. Production of fermented milk product fortified with the AsA-Fe-AmA triple chelate complexes

Milk fermentation was conducted following the method outlined by Sebastián-Nicolas et al. \(^{59}\), with minor modifications. *Lactcaseibacillus rhamnosus* (1 \( \times 10^6 \) CFU), *Lactobacillus acidophilus* (5 \( \times 10^6 \) CFU), and 0.1 mL of 0.5 mol/L AsA-Fe-AmA triple chelate complex solution were added to 150 mL of pasteurized milk. The mixture was stirred and then incubated in a thermostat at 37°C for 12 hours. Milk pasteurization was carried out at 75 °C for 1 min.

2.10. In vivo experiment

At the next stage, the effect of triple chelate complexes, specifically iron ascorbate lysinate, on biochemical parameters in the blood of laboratory *Wistar* rats at modeling ID anemia was investigated. ID anemia was modeled by subcutaneous administration of 0.5 g/kg deferoxamine (Desferal, Novartis Pharma, Switzerland) 2 times with an interval of 3 days.

For a comparative analysis, 6 groups of animals (n = 5) were formed. The first group was remained intact, while the second group served as a control with formed ID anemia. The third and fourth groups were orally administrated the synthesized complex at dosage of 1 mL/200 g and 2 mL/200 g. The fifth and sixth groups were intramuscularly administrated with Ferran drug (Nita-Pharm, Russia) at doses of 1 mL/200 g and 2 mL/200 g, respectively. Ferran is commonly used in veterinary practice for the treatment of ID anemia. Iron preparations were administered on the
12th day of the experiment. Blood was taken for measurement in 24 h after the administration of iron preparations.

Blood for the study was obtained from the heart by piercing the chest wall with a needle with a vacutainer of two types: containing an anticoagulant and containing a gel for separating blood serum. The levels of hemoglobin, erythrocytes, and hematocrit was determined using the Mindray BC-2800 Vet veterinary hematology analyzer (Mindray, Beijing, China). Additionally, the iron content in the blood serum was determined using an automatic biochemical analyzer, the Mindray BS-240 Vet (Mindray, Beijing, China).

2.11. Statistically data processing

The biological and analytical studies were repeated three times and five times, respectively. The parameters were analyzed using STATISTICA for Windows (Statsoft, Tulsa, USA) using one-way ANOVA and Student's T-test (p < 0.05). Microsoft Excel 2010 and Origin software were used to create histograms and graphs based on the collected data.

3. Results and discussion

3.1 Optical properties of the AsA-Fe-AmA triple chelate complexes
Initially, the optical characteristics of the AsA-Fe-AmA triple chelate complexes were investigated. For the investigation, the samples were 100 times diluted in distilled water. Figure 2 shows the acquired absorption spectra.
Figure 2. Absorption spectra of triple chelate complexes of the essential trace element iron with ascorbic acid and essential amino acids: a – L-valine, b – L-leucine, c – L-lysine, d – L-threonine, e – L-isoleucine, f – L-methionine, g – L-tryptophan, h – L-phenylalanine

The absorption spectra (Fig. 2) unveiled that all of the AsA-Fe-AmA triple chelate complexes generated exhibited a maximum absorption band in the absorption spectrum between 552 and 567 nm. Table 2 displays the location of the absorption band maximum ($\lambda_{\text{max}}$) and intensity at $\lambda_{\text{max}}$ ($D_{\text{max}}$).

Table 2. Optical properties of the AsA-Fe-AmA triple chelate complexes

<table>
<thead>
<tr>
<th>AsA-Fe-AmA triple chelate complexe</th>
<th>$\lambda_{\text{max}}$, nm</th>
<th>$D_{\text{max}}$, nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iron ascorbate valinate</td>
<td>559</td>
<td>2.0836</td>
</tr>
<tr>
<td>Iron ascorbate lysinate</td>
<td>567</td>
<td>2.4969</td>
</tr>
<tr>
<td>Iron ascorbate leucinate</td>
<td>552</td>
<td>2.3656</td>
</tr>
<tr>
<td>Iron ascorbate threoninate</td>
<td>554</td>
<td>2.0221</td>
</tr>
<tr>
<td>Iron ascorbate isoleucinate</td>
<td>557</td>
<td>1.2961</td>
</tr>
<tr>
<td>Iron ascorbate methioninate</td>
<td>567</td>
<td>2.6919</td>
</tr>
<tr>
<td>Iron ascorbate tryptophanate</td>
<td>554</td>
<td>1.1281</td>
</tr>
<tr>
<td>Iron ascorbate phenylalaninate</td>
<td>552</td>
<td>0.4324</td>
</tr>
</tbody>
</table>
Analysis of the data presented in Figure 2 and Table 2 revealed that the amino acid in the complex composition influences the location of maximal absorption of the AsA-Fe-AmA triple chelate complex. Specifically, iron ascorbate isoleucinate and phenylalaninate had the lowest $\lambda_{\text{max}}$ (552 nm), while ascorbate methioninate and iron ascorbate lysinate had the highest (567 nm). However, a narrow range of 552-567 nm can attributed to the closely comparable rate of iron concentration and saturation $^{60,61}$.

3.2 Characterization of the AsA-Fe-AmA triple chelate complexes formation

The formation of AsA-Fe-AmA triple chelate complexes was initially explored through computer quantum chemical modeling. Various models were examined, considering the potential formation of a triple chelate complex via amino and carboxyl groups or amino and hydroxyl groups of AmA, as well as hydroxyl groups attached to ascorbic acid's C2, C3, C5, or C6 carbon atoms. Table 3 shows the total energy and chemical rigidity values obtained for the molecular complexes under investigation.

Table 3. Results of computer quantum chemical calculations

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Groups of amino acid</th>
<th>Carbon atoms in ascorbic acid attached with hydroxyl groups</th>
<th>$E$, kcal/mol</th>
<th>$E_{\text{HOMO}}$, eV</th>
<th>$E_{\text{LUMO}}$, eV</th>
<th>$\eta$, eV</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-valin</td>
<td>amino and carboxyl groups</td>
<td>C2 and C3, C2 and C6, C2 and C5, C3 and C6, C3 and C5, C5 and C6</td>
<td>-2338.336, -2338.128, -2338.144, -2338.140, -2338.127, -2338.082</td>
<td>-0.131, -0.114, -0.110, -0.089, -0.082</td>
<td>0.025, 0.045, 0.052, 0.056, 0.065</td>
<td>0.078, 0.080, 0.081, 0.073, 0.074</td>
</tr>
<tr>
<td>L-leucine</td>
<td>amino and carboxyl groups</td>
<td>C2 and C3, C2 and C6, C2 and C5, C3 and C6, C3 and C5, C5 and C6</td>
<td>-2377.402, -2377.021, -2377.204, -2377.010, -2377.124, -2377.080</td>
<td>-0.142, -0.154, -0.105, -0.186, -0.095</td>
<td>0.026, 0.042, 0.035, 0.018, 0.053</td>
<td>0.084, 0.098, 0.070, 0.102, 0.074</td>
</tr>
<tr>
<td>L-isoleucine</td>
<td>amino and carboxyl groups</td>
<td>C2 and C3, C2 and C6, C2 and C5, C3 and C6, C3 and C5, C5 and C6</td>
<td>-2377.365, -2377.142, -2377.137, -2377.162, -2377.078, -2377.083</td>
<td>-0.129, -0.175, -0.102, -0.110, -0.096</td>
<td>0.015, 0.042, 0.036, 0.054, 0.041</td>
<td>0.072, 0.109, 0.069, 0.082, 0.069</td>
</tr>
</tbody>
</table>
Computer quantum chemical modeling revealed that the molecule complex models are not only energetically favorable but also chemically stable (0.058 < η < 0.113 eV) \(^{62}\). The interaction between AmA and AsA was found to occurs through the amino and carboxyl groups of AmA and hydroxyl groups attached to C2 and C3 carbon atoms of AsA (Figure 3). This configuration was favored due to its minimal total value energy compared to other configurations \(^{63,64}\).
Figure 3. Possible variants of the AsA-Fe-AmA triple chelate complexes formation on example of iron ascorbate leucinate (hydroxyl groups involved in the complex formation are marked by circles).

The Supplementary (Figures S1-S60) contains models of molecular complexes, electron density distributions, electron density distribution gradients, and visualizations of the highest occupied molecular orbital (HOMO) and lowest occupied molecular orbital (LUMO) of the AsA-Fe-AmA triple chelate complexes.

To validate the results of computer quantum chemical modeling on AsA-Fe-AmA triple chelate complexes, FTIR spectroscopy was employed. Figure 4 and Supplementary (Figures S61-S62) exhibit the acquired data.

The IR spectra of the AsA-Fe-AmA triple chelate complexes (Figure 4) revealed valence variations for the -CO$_2^-$, NH$_3^+$, OH, CH$_2$, and CH$_3$ groups between 2500 and 3500 cm$^{-1}$. Additionally, the infrared spectra show bands typical of AsA:
the bands at 634, 719, and 1755 cm\(^{-1}\) correspond to fluctuations of C=O; the band at 1670 cm\(^{-1}\) corresponds to vibrations of C=C in the C2 and C3 atoms of AsA; the bands at 1498 cm\(^{-1}\), 1365 cm\(^{-1}\), 1197 cm\(^{-1}\), and 990 cm\(^{-1}\) corresponded to the deformation vibrations of the CH\(_2\) group\(^{65,66}\). Furthermore, up to 1300 cm\(^{-1}\), there are deformation variations of -CH\(_2\) and -CH\(_3\) groups, which are typical of AmA\(^{67}\).

Figure 4. The IR spectra of the AsA-Fe-AmA triple chelate complexes: 1 – L-valine, 2 – L-isoleucine, 3 – L-leucine, 4 – L-phenylalanine, 5 – L-tryptophan, 6 – L-methionine, 7 – L-lysine, 8 – L-threonine

It is worth noting that the IR spectra of the AsA-Fe-AmA triple chelate complexes show a drop in the strength of a number of bands.

- AmA's NH\(_3^+\) group deformation fluctuations occur in the 1485-1550 cm\(^{-1}\) range;
- COO\(^-\) group fluctuations occur in the 1300-1330 cm\(^{-1}\) range;
- AsA's C-OH group deformation vibrations occur at 1278 and 1448 cm\(^{-1}\);
- AsA's C-OH group deformation vibrations occur at 1330 cm\(^{-1}\).

The analysis of the IR spectra showed bands that are not indicative of AsA or AmA:

- 1160-1170 cm\(^{-1}\) corresponds to C-O-Fe variations\(^{68}\);
- in the range 795-805 cm\(^{-1}\), which corresponds to Fe-N variations\(^{69}\).

Consequently, the AsA-Fe-AmA triple chelate complexes are formed via the AsA OH group and the AmA COO\(^-\) and NH\(_3^+\) groups, respectively. The interaction between Fe and the OH group of AsA results in the formation of a C-O-Fe bond in
the 1160-1170 cm\(^{-1}\) range and a decrease in the intensity of the bands C-O-H (1278 and 1448 cm\(^{-1}\)) and C-OH (1330 cm\(^{-1}\)).

It was discovered that the interaction between Fe and the COO\(^{-}\) group of AmA results in the production of a C-O-Fe bond in the range 1160-1170 cm\(^{-1}\) accompanied by reduction in the strength of the COO\(^{-}\) group band (1300-1330 cm\(^{-1}\)). Simultaneously, the interaction between Fe and the NH\(_3^+\) group of AmA results in the production of a Fe-N bond in the range 795-805 cm\(^{-1}\), along with a decrease in the strength of the NH\(_3^+\) group’s band of deformation vibrations (1485-1550 cm\(^{-1}\)). Figure 8 depicts the formation of triple chelate complexes of the necessary trace metal iron, ascorbic acid, and essential amino acids.

Hence, FTIR spectroscopy results are consistent with computer quantum chemical modeling. Drawing from these findings, a technique for the creation of AsA-Fe-AmA triple chelate complexes was devised and illustrated in Figure 5.

![Figure 5](image)

**Figure 5.** Scheme for the formation of the AsA-Fe-AmA triple chelate complexes on example of iron ascorbate lysinate

### 3.3. Stability of the AsA-Fe-AmA triple chelate complexes

In addition to comprehending the formation process of the AsA-Fe-AmA triple chelate complexes, it is critical to understand their stability. To achieve this, a
multifactorial experiment was conducted, incorporating three input parameters: pH, temperature (T), and exposure period. The output parameter was specified as the change in optical density (ΔD):

$$\Delta D = D_0 - D_{\text{pH, T, } \tau}$$  \hspace{1cm} (1)

where:

- $D_0$ – the value of the optical density at $\lambda_{\text{max}}$ in the absorption spectrum of the complex after obtaining.
- $D_{\text{pH, T, } \tau}$ – the value of the optical density at $t \lambda_{\text{max}}$ in the absorption spectrum of the complex at given values of pH, T and $\tau$.

The collected data underwent mathematical and statistical analysis, as illustrated in Figures 9 and Supplementary section, which displays the obtained dependencies. The examination of the acquired dependencies revealed that the stability of the AsA-Fe-AmA triple chelate complexes is significantly influenced by all studied factors. Increases in pH, T, and $\tau$ lead to a rise in $\Delta D$, indicating complicated decay. Conversely, the highest $\Delta D$ values were recorded at pH = 8-11, T = 75-95 °C, and $\tau = 20\text{--}25$ min. The lowest $\Delta D$ values were found at pH = 3-7, T = 20-75 °C, and $\tau = 5\text{--}15$ min, indicating the complexes' stability in this range of parameters (Figure 6 and Supplementary (Figures S63-S69)).

Analysis of these dependencies revealed that the stability of the AsA-Fe-AmA triple chelate complexes is significantly influenced by all of the factors investigated. Increases in pH, temperature (T), and exposure period ($\tau$) lead to a rise in $\Delta D$, indicating complex decay processes. The highest $\Delta D$ values were recorded at pH = 8-11, T = 75-95 °C, and $\tau = 20\text{--}25$ min. Conversely, the lowest $\Delta D$ values were found at pH = 3-7, T = 20-75 °C, and $\tau = 5\text{--}15$ min, indicating greater stability of the complexes within this parameter range (Figure 6 and Supplementary (Figures S63-S69)).
Thus, it can be concluded that the newly synthesized AsA-Fe-AmA triple chelate complexes are appropriate for fortification of food items with a pH range of 3-7 and have not been exposed to heat treatment at temperatures over 70 °C for more than 15 minutes. Pasteurized milk and fermented dairy products derived from it (kefir, ryazhenka, yogurt, etc.) were identified as a suitable food matrix that could be included in a daily diet, as supported by multiple recent studies. However, these compounds may have a considerable impact on the stable dispersion system of milk and fermented dairy products. As a result, the effect of the AsA-Fe-AmA triple chelate complexes on the distributed content of milk should be thoroughly investigated.

Figure 6. Stability assessment of the AsA-Fe-AmA triple chelate complexes: dependences of changes in optical density on pH and T (a), pH and τ (b), T and τ (c); absorption spectra of samples in the region with the largest change in optical density (d) and with the smallest change in optical density (e) (iron ascorbate lysinate)

3.4 In vivo experiment
At the next stage, the effect of a complex of triple chelate complexes, in particular, iron ascorbate lysinate, on the biochemical parameters of blood of laboratory Wistar rats was investigated. The results are presented in table 4.

**Table 4. Biochemical parameters of blood of laboratory rats with modelling ID anemia**

<table>
<thead>
<tr>
<th>Index</th>
<th>Group 1 (Intact)</th>
<th>Group 2 (Control with ID anemia)</th>
<th>Group 3 (1 mL/200 g AsA-Fe-AmA)</th>
<th>Group 4 (2 mL/200 g AsA-Fe-AmA)</th>
<th>Group 5 (1 mL/200 g Ferran)</th>
<th>Group 6 (2 mL/200 g Ferran)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin, g/L</td>
<td>16.19±0.51</td>
<td>10.43±0.48</td>
<td>10.65±0.50</td>
<td>10.48±0.48</td>
<td>10.42±0.51</td>
<td>10.46±0.54</td>
</tr>
<tr>
<td>Erythrocytes, ×10^{12}/L</td>
<td>11.60±0.14</td>
<td>7.45±0.50</td>
<td>7.47±0.48</td>
<td>7.43±0.51</td>
<td>7.46±0.49</td>
<td>7.45±0.47</td>
</tr>
<tr>
<td>Hematocrit, %</td>
<td>35.25±1.15</td>
<td>23.19±0.93</td>
<td>23.24±0.87</td>
<td>23.15±0.76</td>
<td>23.42±0.77</td>
<td>23.31±0.80</td>
</tr>
<tr>
<td>Fe, mmol/L</td>
<td>26.3±0.8</td>
<td>16.1±1.3</td>
<td>27.8±1.0</td>
<td>30.1±0.9</td>
<td>26.0±1.1</td>
<td>28.9±1.2</td>
</tr>
</tbody>
</table>

Table 4 shows that the level of hemoglobin, erythrocytes and hematocrit in all groups did not differ statistically. Notably, according to the literature data, blood parameters of laboratory animals (hemoglobin, erythrocytes and hematocrit levels) should recover in 30 days. At the same time, serum iron values after a single application of AsA-Fe-AmA reached reference values within 24 h after administration. There was a statistically significant difference in the level of iron in blood of laboratory rats. After administration of AsA-Fe-AmA, iron level increased by 10.7 mmol/L and 14.0 mmol/L in group 3 and group 4, respectively, compared to control group. Notably, AsA-Fe-AmA increased the iron level in blood by 1.5 mmol/L (group 3) and 3.8 mmol/L (group 4) compared with intact group without ID anemia modelling. Moreover, there was not statistically differences between effect of AsA-Fe-AmA and Ferran drug which is commonly used in veterinary practice for the treatment of ID anemia. Thus, AsA-Fe-AmA balanced iron level in blood of laboratory rats with modelling ID anemia in 24 h after oral administration which is compared with effectiveness of commercial drugs.
3.5. Effect of the AsA-Fe-AmA triple chelate complexes on the dispersed composition of milk

Table 5 shows the effects of the AsA-Fe-AmA triple chelate complexes on milk’s dispersed composition, encompassing parameters such as titrated acidity (TA), electrical conductivity ($\delta$), pH, average hydrodynamic radius (R), and $\zeta$-potential at various complex concentrations.
Table 5. Effect of the AsA-Fe-AmA triple chelate complexes on the dispersed composition of milk

<table>
<thead>
<tr>
<th>Concentration, mol/L</th>
<th>Parameters</th>
<th>Iron ascorbate lysinate</th>
<th>Iron ascorbate leucinate</th>
<th>Iron ascorbate isoleucinate</th>
<th>Iron ascorbate valinate</th>
<th>Iron ascorbate phenylalaninate</th>
<th>Iron ascorbate threoninate</th>
<th>Iron ascorbate tryptophanate</th>
<th>Iron ascorbate methioninate</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05</td>
<td>R, nm</td>
<td>314 ± 20</td>
<td>248 ± 22</td>
<td>429 ± 37</td>
<td>988 ± 78</td>
<td>132 ± 12</td>
<td>441 ± 34</td>
<td>89 ± 9</td>
<td>381 ± 34</td>
</tr>
<tr>
<td></td>
<td>δ, S</td>
<td>0.40 ± 0.5</td>
<td>0.94 ± 0.5</td>
<td>0.77 ± 0.5</td>
<td>0.99 ± 0.5</td>
<td>1.00 ± 0.5</td>
<td>1.15 ± 0.5</td>
<td>1.08 ± 0.5</td>
<td>0.81 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>ζ-potential, mV</td>
<td>-1.08 ± 1.00</td>
<td>-0.48 ± 1.00</td>
<td>1.07 ± 1.00</td>
<td>-1.04 ± 1.00</td>
<td>-0.97 ± 1.00</td>
<td>-0.44 ± 1.00</td>
<td>-1.91 ± 1.00</td>
<td>-0.2 ± 1.00</td>
</tr>
<tr>
<td></td>
<td>pH</td>
<td>6.62 ± 0.05</td>
<td>6.45 ± 0.05</td>
<td>6.44 ± 0.05</td>
<td>6.47 ± 0.05</td>
<td>6.12 ± 0.05</td>
<td>6.01 ± 0.05</td>
<td>5.42 ± 0.05</td>
<td>6.48 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>TA, °T</td>
<td>55 ± 1</td>
<td>55 ± 1</td>
<td>37 ± 1</td>
<td>37 ± 1</td>
<td>20 ± 1</td>
<td>19 ± 1</td>
<td>17 ± 1</td>
<td>18 ± 1</td>
</tr>
<tr>
<td>0.005</td>
<td>R, nm</td>
<td>44 ± 5</td>
<td>45 ± 5</td>
<td>49 ± 5</td>
<td>69 ± 6</td>
<td>50 ± 5</td>
<td>51 ± 5</td>
<td>50 ± 5</td>
<td>79 ± 7</td>
</tr>
<tr>
<td></td>
<td>δ, S</td>
<td>0.70 ± 0.5</td>
<td>0.70 ± 0.5</td>
<td>0.74 ± 0.5</td>
<td>0.94 ± 0.5</td>
<td>1.39 ± 0.5</td>
<td>0.93 ± 0.5</td>
<td>0.76 ± 0.5</td>
<td>0.92 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>ζ-potential, mV</td>
<td>-0.39 ± 1.00</td>
<td>0.3 ± 1.00</td>
<td>-0.45 ± 1.00</td>
<td>-0.26 ± 1.00</td>
<td>-0.56 ± 1.00</td>
<td>-0.27 ± 1.00</td>
<td>-0.44 ± 1.00</td>
<td>0.32 ± 1.00</td>
</tr>
<tr>
<td></td>
<td>pH</td>
<td>6.68 ± 0.05</td>
<td>6.66 ± 0.05</td>
<td>6.69 ± 0.05</td>
<td>6.75 ± 0.05</td>
<td>6.7 ± 0.05</td>
<td>6.73 ± 0.05</td>
<td>6.76 ± 0.05</td>
<td>6.76 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>TA, °T</td>
<td>19 ± 1</td>
<td>19 ± 1</td>
<td>20 ± 1</td>
<td>17 ± 1</td>
<td>19 ± 1</td>
<td>20 ± 1</td>
<td>19 ± 1</td>
<td>18 ± 1</td>
</tr>
<tr>
<td>0.0005</td>
<td>R, nm</td>
<td>36 ± 4</td>
<td>31 ± 4</td>
<td>38 ± 4</td>
<td>49 ± 5</td>
<td>36 ± 4</td>
<td>37 ± 4</td>
<td>35 ± 4</td>
<td>41 ± 4</td>
</tr>
<tr>
<td></td>
<td>δ, S</td>
<td>0.91 ± 0.5</td>
<td>0.80 ± 0.5</td>
<td>0.79 ± 0.5</td>
<td>0.76 ± 0.5</td>
<td>1.02 ± 0.5</td>
<td>2.03 ± 0.5</td>
<td>0.79 ± 0.5</td>
<td>0.71 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>ζ-potential, mV</td>
<td>0.23 ± 1.00</td>
<td>-0.94 ± 1.00</td>
<td>-0.93 ± 1.00</td>
<td>0.14 ± 1.00</td>
<td>0.52 ± 1.00</td>
<td>0.42 ± 1.00</td>
<td>-0.70 ± 1.00</td>
<td>-0.84 ± 1.00</td>
</tr>
<tr>
<td></td>
<td>pH</td>
<td>6.76 ± 0.05</td>
<td>6.76 ± 0.05</td>
<td>6.75 ± 0.05</td>
<td>6.78 ± 0.05</td>
<td>6.73 ± 0.05</td>
<td>6.75 ± 0.05</td>
<td>6.74 ± 0.05</td>
<td>6.8 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>TA, °T</td>
<td>17 ± 1</td>
<td>19 ± 1</td>
<td>18 ± 1</td>
<td>17 ± 1</td>
<td>18 ± 1</td>
<td>19 ± 1</td>
<td>18 ± 1</td>
<td>18 ± 1</td>
</tr>
<tr>
<td>0.00005</td>
<td>R, nm</td>
<td>31 ± 3</td>
<td>28 ± 3</td>
<td>32 ± 3</td>
<td>33 ± 3</td>
<td>36 ± 4</td>
<td>31 ± 3</td>
<td>29 ± 3</td>
<td>31 ± 3</td>
</tr>
<tr>
<td></td>
<td>δ, S</td>
<td>0.73 ± 0.5</td>
<td>0.74 ± 0.5</td>
<td>0.85 ± 0.5</td>
<td>0.78 ± 0.5</td>
<td>0.85 ± 0.5</td>
<td>1.77 ± 0.5</td>
<td>0.92 ± 0.5</td>
<td>1.03 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>ζ-potential, mV</td>
<td>-0.77 ± 1.00</td>
<td>0.85 ± 1.00</td>
<td>0.66 ± 1.00</td>
<td>0.61 ± 1.00</td>
<td>0.45 ± 1.00</td>
<td>0.39 ± 1.00</td>
<td>-1.36 ± 1.00</td>
<td>0.75 ± 1.00</td>
</tr>
<tr>
<td></td>
<td>pH</td>
<td>6.77 ± 0.05</td>
<td>6.78 ± 0.05</td>
<td>6.76 ± 0.05</td>
<td>6.79 ± 0.05</td>
<td>6.80 ± 0.05</td>
<td>6.75 ± 0.05</td>
<td>6.77 ± 0.05</td>
<td>6.79 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>TA, °T</td>
<td>21 ± 1</td>
<td>17 ± 1</td>
<td>18 ± 1</td>
<td>17 ± 1</td>
<td>18 ± 1</td>
<td>20 ± 1</td>
<td>18 ± 1</td>
<td>18 ± 1</td>
</tr>
</tbody>
</table>

Control

<table>
<thead>
<tr>
<th>Parameters</th>
<th>R, nm</th>
<th>δ, S</th>
<th>ζ-potential, mV</th>
<th>pH</th>
<th>TA, °T</th>
</tr>
</thead>
</table>
| R – average hydrodynamic radius, δ – electrical conductivity, TA – titrated acidity
| * – X ± standard error of device (N = 3)
According to Table 5, 0.05 mol/L AsA-Fe-AmA triple chelate complexes induced the coagulation of milk proteins, leading to separation and precipitation of milk fraction. At this concentration, samples with iron ascorbate valinate had the most significant increase in the average hydrodynamic radius of casein micelles (980 nm vs. 30 nm in the control sample). Samples with iron ascorbate tryptophanate had the highest titrated acidity of 55 °T (18 °T in the control sample) and the lowest pH (5.42 vs. 6.78 in the control sample). Electrical conductivity and ζ-potential remained stable. Figure 7 depicts a histogram of the average hydrodynamic radius of casein micelles in milk samples with 0.05 mol/L iron ascorbate valinate. It is noteworthy that the similar tendency of casein micelle growth was reported in our earlier study on milk fortification with zinc lysinate riboflavinate at concentrations greater than 0.05 mol/L.

At concentrations of 0.005 mol/L, 0.0005 mol/L, and 0.00005 mol/L, the samples exhibited no significant alterations. Casein micelles maintained an average hydrodynamic radius of 27–79 nm, titrated acidity of 17 – 21 °T, and a pH range of 6.66–6.80. Electrical conductivity and ζ-potential remained stable. Figure 8 depicts
a histogram of the average hydrodynamic radius of casein micelles in milk samples with 0.005 mol/L iron ascorbate valinate.

Figure 8. Histogram of the average hydrodynamic radius of casein micelles in milk sample with 0.005 mol/L iron ascorbate valinate

The analysis of the acquired data revealed that milk should be fortified with the AsA-Fe-AmA triple chelate complexes at concentrations of 0.005 mol/L or less, corresponding to a dilution of 1:100 or less (Figure 9).

Figure 9. Scheme of the effect of the AsA-Fe-AmA triple chelate complexes on casein micelles
3.6 Antioxidant activity and sensory properties of milk fortified with the AsA-Fe-AmA triple chelate complexes

In the subsequent stage of the experiment, the antioxidant activity (AoA) of fortified milk was investigated. The data obtained are presented in table 6.

Table 6. Antioxidant activity of milk fortified the AsA-Fe-AmA triple chelate complexes

<table>
<thead>
<tr>
<th>Samples</th>
<th>D (blank sample), a. u.</th>
<th>D (sample), a. u.</th>
<th>AoA, %</th>
<th>AoA, mg TE/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk with iron ascorbate lysinate</td>
<td>0.700</td>
<td>0.638</td>
<td>17.7</td>
<td>0.66</td>
</tr>
<tr>
<td>Milk with iron ascorbate phenylalaninate</td>
<td>0.694</td>
<td>0.609</td>
<td>24.5</td>
<td>0.92</td>
</tr>
<tr>
<td>Milk with iron ascorbate isoleucinate</td>
<td>0.697</td>
<td>0.628</td>
<td>19.8</td>
<td>0.74</td>
</tr>
<tr>
<td>Milk with iron ascorbate tryptophanate</td>
<td>0.707</td>
<td>0.645</td>
<td>17.5</td>
<td>0.66</td>
</tr>
<tr>
<td>Milk with iron ascorbate methioninate</td>
<td>0.697</td>
<td>0.645</td>
<td>14.9</td>
<td>0.56</td>
</tr>
<tr>
<td>Milk with iron ascorbate valinate</td>
<td>0.704</td>
<td>0.645</td>
<td>16.8</td>
<td>0.63</td>
</tr>
<tr>
<td>Milk with iron ascorbate threoninate</td>
<td>0.702</td>
<td>0.652</td>
<td>14.2</td>
<td>0.53</td>
</tr>
<tr>
<td>Milk with iron ascorbate leucinate</td>
<td>0.727</td>
<td>0.675</td>
<td>14.7</td>
<td>0.55</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>0.713</td>
<td>0.640</td>
<td>20.3</td>
<td>0.76</td>
</tr>
<tr>
<td>1 mg trolox</td>
<td>0.727</td>
<td>0.533</td>
<td>26.7</td>
<td>1</td>
</tr>
<tr>
<td>Control milk sample</td>
<td>0.731</td>
<td>0.702</td>
<td>7.9</td>
<td>0.30</td>
</tr>
</tbody>
</table>

Table 6 demonstrates that the AsA-Fe-AmA triple chelate complexes improved the AoA of enriched milk samples. The most significant impact was found with the use of iron ascorbate phenylalaninate (0.92 mg TE/mL), while the lowest effect was noted with the use of iron ascorbate threoninate (0.53 mg TE/mL), still surpassing the AoA value in the control sample (0.30 mg TE/mL). Interestingly, iron ascorbate phenylalaninate exhibited higher AoA than AsA. These results align with the well-known AoA of AsA and AmA and their efficacy in milk fortification. Iron complexes are known to significantly influence on the sensory qualities of milk and dairy products, which warrants further investigation. García et al. reported that iron fortification at different concentrations markedly affected the sensory acceptability of milk. Abdulghani et al. observed the same outcomes. Siddique and Park produced and tested cheese enriched with ordinary ferrous...
sulfate and big microencapsulated ferrous sulfate. **While the iron-fortified cheese exhibited improved functional and structural qualities, it received lower taste ratings, posing a challenge for industrial production.** Arce and Ustunol also found that iron-fortified cheese displayed poor sensory characteristics, rating lower than control samples in appearance, texture, taste, and overall acceptability.

**Hence,** iron fortification of milk might result in considerable changes in sensory characteristics, decreasing its acceptability. Thus, sensory study of milk enriched with AsA-Fe-AmA triple chelate complexes was critical. Table 7 presents the sensory evaluation results.

Table 7. Sensory characteristics of milk fortified with the AsA-Fe-AmA triple chelate complexes

<table>
<thead>
<tr>
<th>Sample</th>
<th>General description</th>
<th>Total value *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk with iron (II) sulfate</td>
<td>Clean, pleasant, slightly sweet</td>
<td>4.81</td>
</tr>
<tr>
<td>Milk with iron ascorbate lysinate</td>
<td>Slightly pronounced unclean, foreign smell and taste</td>
<td>4.40</td>
</tr>
<tr>
<td>Milk with iron ascorbate valinate</td>
<td>Slightly pronounced unclean, foreign smell and taste</td>
<td>3.18</td>
</tr>
<tr>
<td>Milk with iron ascorbate isoleucinate</td>
<td>Slightly pronounced unclean, foreign smell and taste</td>
<td>3.63</td>
</tr>
<tr>
<td>Milk with iron ascorbate threoninate</td>
<td>Insufficiently pronounced, empty, without foreign odors and flavors</td>
<td>3.96</td>
</tr>
<tr>
<td>Milk with iron ascorbate methioninate</td>
<td>Pronounced unclean, peculiar smell, salty taste</td>
<td>2.83</td>
</tr>
<tr>
<td>Milk with iron ascorbate tryptophanate</td>
<td>Clean, pleasant, slightly sweet</td>
<td>4.70</td>
</tr>
<tr>
<td>Milk with iron ascorbate phenylalaninate</td>
<td>Clean, pleasant, slightly sweet</td>
<td>4.65</td>
</tr>
<tr>
<td>Milk with iron ascorbate leucinate</td>
<td>Insufficiently pronounced, empty, without foreign odors and flavors</td>
<td>4.02</td>
</tr>
<tr>
<td>Control milk sample</td>
<td>Clean, pleasant, slightly sweet</td>
<td>4.81</td>
</tr>
</tbody>
</table>

* – weighted average value (N = 10)

The sensory evaluation revealed that milk samples fortified with iron (II) sulfate, iron ascorbate tryptophanate, and iron ascorbate phenylalaninate had a clean, pleasant, slightly sweet taste and odor. **However,** milk samples fortified with other AsA-Fe-AmA triple chelate complexes had changes in taste and odor, resulting in lower overall sensory acceptability scores. **Consequently,** iron ascorbate tryptophanate and iron ascorbate phenylalaninate were identified as the best
complexes for milk fortification based on sensory acceptability. They might warrant further investigated as a viable substance for food fortification in iron deficiency anemia.

3.7. Effect of the AsA-Fe-AmA triple chelate complexes on lactic acid bacteria

Recent studies have demonstrated the antibacterial activity of iron complexes used for milk fortification. For instance, Helmyati et al. 86 fortified milk with NaFeEDTA and FeSO₄ and recorded antibacterial action against *E. coli* and *S. aureus*, as well as a reduction in total enterobacteriaceae over a four-week monitoring period. Similarly, Harouna et al. 87 found iron-saturated bovine lactoferrin reduced the viability of *C. sakazakii* in whey. Consequently, when producing fermented dairy products from milk enriched with AsA-Fe-AmA triple chelate complexes, it is critical to understand how these complexes interact with bacteria, as their antibacterial impact may inhibit milk fermentation. To investigate the effect of the AsA-Fe-AmA triple chelate complexes on lactic acid bacteria, solutions of the complexes were prepared at a concentration of 0.05 mol/L (A), 0.005 mol/L (B), 0.0005 mol/L (C) and 0.00005 mol/L (D) for research purposes. Subsequently, 100 µL of *Lacticaseibacillus rhamnosus* suspensions were added to test tubes with the AsA-Fe-AmA triple chelate complexes solutions and plated on Petri dishes with agar-agar. The Petri dishes were then placed in a thermostat (Binder GmbH, Tuttlingen, Germany) at 37 °C for 24 hours. The obtained photos of Petri dishes are shown in Figure 10.
Figure 10. Photographs of Petri dishes with *Lacticaseibacillus rhamnosus* seeded in agar-agar medium with iron ascorbate threoninate (2), iron ascorbate valinate (2), iron ascorbate methioninate (3), iron ascorbate lysinate (4), iron ascorbate leucinate (5), iron ascorbate phenylalaninate (6), iron ascorbate isoleucinate (7), iron ascorbate tryptophanate (8) at different concentrations: 0.05 mol/L (A), 0.005 mol/L (B), 0.0005 mol/L (C) and 0.00005 mol/L (D).

The colony-forming units (CFU) value was determined for all samples and presented in Table 8.

Table 8. Effect of the AsA-Fe-AmA triple chelate complexes on *Lacticaseibacillus rhamnosus*

<table>
<thead>
<tr>
<th>AsA-Fe-AmA triple chelate complexes</th>
<th>CFU, ×10^6 in 1 mL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.05 mol/L</td>
</tr>
<tr>
<td>iron ascorbate threoninate</td>
<td>0.82</td>
</tr>
<tr>
<td>iron ascorbate valinate</td>
<td>0.48</td>
</tr>
<tr>
<td>iron ascorbate methioninate</td>
<td>0.80</td>
</tr>
<tr>
<td>iron ascorbate lysinate</td>
<td>0.75</td>
</tr>
<tr>
<td>iron ascorbate leucinate</td>
<td>0.76</td>
</tr>
<tr>
<td>iron ascorbate phenylalaninate</td>
<td>0.80</td>
</tr>
<tr>
<td>iron ascorbate isoleucinate</td>
<td>0.79</td>
</tr>
<tr>
<td>iron ascorbate tryptophanate</td>
<td>0.77</td>
</tr>
<tr>
<td>Control</td>
<td>1.2</td>
</tr>
</tbody>
</table>


According to the findings in Table 8, the concentration and type of AsA-Fe-AmA triple chelate complexes exert a substantial impact on the growth and development of *Lacticaseibacillus rhamnosus* lactic acid cultures. However, at a concentration of 0.05 mol/L, CFU varies from $0.75 \times 10^6$ to $0.82 \times 10^6$ in 1 mL, with no significant difference between them. At a concentration of 0.005 mol/L, CFU varies from 0 to $1.56 \times 10^6$ per 1 mL. Iron ascorbate valinate was the sole complex that had a detrimental influence on the growth and development of *Lacticaseibacillus rhamnosus*. Notably, decreasing quantities of iron ascorbate threoninate, iron ascorbate lysinate, ascorbate leucinate, and iron ascorbate isoleucinate resulted in a rise in the CFU value.

Similarly, iron ascorbate methioninate, iron ascorbate phenylalaninate, and iron ascorbate tryptophanate showed no significant influence on concentration. At 0.0005 mol/L, iron ascorbate threoninate, iron ascorbate methioninate, iron ascorbate lysinate, and iron ascorbate tryptophanate improved the growth and development of *Lacticaseibacillus rhamnosus* compared to the control sample. The findings are significant as the complexes can increase milk fermentation while fortifying the end product with iron.

### 3.8. Production of fermented milk product fortified with the AsA-Fe-AmA triple chelate complexes

The fermented dairy product samples fortified with AsA-Fe-AmA triple chelate complexes underwent testing for titrated acidity, pH, and viscosity. The results are shown in Table 9.

#### Table 9. Physical and chemical properties of fermented milk product fortified with the AsA-Fe-AmA triple chelate complexes

<table>
<thead>
<tr>
<th>AsA-Fe-AmA triple chelate complexes</th>
<th>Titrated acidity, $\circ T^*$</th>
<th>pH $^*$</th>
<th>viscosity, Pa·s $^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>iron ascorbate lysinate</td>
<td>66 ± 1</td>
<td>$4.39 \pm 0.05$</td>
<td>$1896 \pm 132$</td>
</tr>
<tr>
<td>iron ascorbate isoleucinate</td>
<td>104 ± 1</td>
<td>$4.37 \pm 0.05$</td>
<td>$2313 \pm 161$</td>
</tr>
<tr>
<td>iron ascorbate leucinate</td>
<td>104 ± 1</td>
<td>$4.40 \pm 0.05$</td>
<td>$2075 \pm 145$</td>
</tr>
<tr>
<td>iron ascorbate methioninate</td>
<td>67 ± 1</td>
<td>$4.38 \pm 0.05$</td>
<td>$2018 \pm 142$</td>
</tr>
<tr>
<td>iron ascorbate valinate</td>
<td>57 ± 1</td>
<td>$4.39 \pm 0.05$</td>
<td>$1979 \pm 139$</td>
</tr>
</tbody>
</table>
Table 9 illustrates that fortifying fermented milk products with AsA-Fe-AmA triple chelate complexes resulted in a significant increase in titrated acidity across the samples. Among them, sample containing iron ascorbate tryptophanate had the highest titrated acidity value (193 °T). Interestingly, the pH of the samples remained unaffected by the AsA-Fe-AmA triple chelate complexes. There was minimal change in viscosity compared to the control sample, except for samples with iron ascorbate isoleucinate, which rose at 504 Pa·s. The established trend of increasing titrated acidity and slightly increasing viscosity aligns with the previous findings in studies involving milk, yogurt, cheese, and similar products., etc.

The sensory assessment of a fermented milk product enriched with the AsA-Fe-AmA triple chelate complexes was conducted at the end of the experiment (Table 10).

Table 10. Sensory characteristics of fermented milk product fortified with the AsA-Fe-AmA triple chelate complexes.

<table>
<thead>
<tr>
<th>Sample</th>
<th>General description</th>
<th>Total value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>iron (II) sulfate</td>
<td>The presence of lumps, creamy color, whey odor and taste, lumpy structure, fluid</td>
<td>4.00</td>
</tr>
<tr>
<td>iron ascorbate lysinate</td>
<td>The presence of lumps, creamy color, whey smell and taste, lumpy structure, fluid</td>
<td>4.40</td>
</tr>
<tr>
<td>iron ascorbate valinate</td>
<td>The presence of lumps, creamy color, whey smell and taste, lumpy structure, fluid</td>
<td>3.40</td>
</tr>
<tr>
<td>iron ascorbate isoleucinate</td>
<td>The presence of lumps, creamy color, whey smell and taste, lumpy structure, fluid</td>
<td>4.00</td>
</tr>
<tr>
<td>iron ascorbate threoninate</td>
<td>The presence of lumps, creamy color, whey smell and taste, lumpy structure, fluid</td>
<td>3.35</td>
</tr>
<tr>
<td>iron ascorbate methioninate</td>
<td>Lumps-free, creamy color, uncharacteristic odor and taste, homogeneous fluid structure</td>
<td>3.30</td>
</tr>
<tr>
<td>iron ascorbate tryptophanate</td>
<td>The presence of lumps, creamy color, whey smell and taste, lumpy structure, fluid</td>
<td>3.50</td>
</tr>
<tr>
<td>iron ascorbate phenylalaninate</td>
<td>Lumps-free, creamy color, uncharacteristic odor and taste, homogeneous fluid structure</td>
<td>3.25</td>
</tr>
<tr>
<td>iron ascorbate leucinate</td>
<td>The presence of lumps, creamy color, whey smell and taste, lumpy structure, fluid</td>
<td>4.00</td>
</tr>
</tbody>
</table>
The sensory evaluation revealed that fermented milk product samples fortified with the AsA-Fe-AmA triple chelate complexes primarily exhibited a creamy color, lumps in the composition, a whey odor and taste, lumpiness, and a fluid structure, except for those fortified with iron ascorbate phenylalaninate and iron ascorbate methioninate, which received the lowest total value. It is crucial to highlight samples fortified with iron ascorbate phenylalanine and iron ascorbate methioninate, emitted a distinct odor characteristic of the amino acids phenylalanine and methionine. The overall value was rather low in samples containing iron ascorbate threoninate, iron ascorbate valinate, and iron ascorbate tryptophanate. Sensory acceptance plays a vital role in developing new food products as it directly influences customer behavior.88,89

The results revealed that iron ascorbate lysinate boosts the growth and development of lactic acid bacteria *Lacticaseibacillus rhamnosus*, possesses strong antioxidant activities, and maintains dispersed characteristics of the dispersed phase of milk. Fortification of fermented milk product with iron ascorbate lysinate has no negative effect on taste and odor of the resulting product. As a result, based on these findings, iron ascorbate lysinate was chosen as the optimal AsA-Fe-AmA triple chelate complex for fortifying fermented milk product and can be further investigated as a potential molecule for addressing iron deficiency anemia food through food fortification.

In summary, the developed triple chelate complexes have been observed to be effective for fortification of dairy and fermented milk products, thereby preventing ID and ID anemia. It is noteworthy that these complexes can be used not only in the food industry, but also in medicine, pharmacy, and veterinary as dietary supplements for prevention of ID and ID anemia in humans and animals.

**Conclusions**
This work marks the first study of novel AsA-Fe-AmA triple chelate complexes. Analysis of their optical properties revealed that all the collected samples of AsA-Fe-AmA triple chelate complexes exhibit a maximum absorption band in the absorption spectrum between 552 and 567 nm. Computer quantum chemical modeling highlighted molecular interactions involving amino and carboxyl groups of AmA and hydroxyl groups linked to the C2 and C3 carbon atoms of AsA. FTIR spectroscopy elucidated the mechanism of the AsA-Fe-AmA triple chelate complexes. Study of the stability of the AsA-Fe-AmA triple chelate complexes at various technical parameters suggested their suitability for fortify food items with pH 3-7, not subjected to heat treatment exceeding 75 °C for more than 15 minutes.

It is important to note that AsA-Fe-AmA triple chelate complexes increases serum iron levels in rats with modelled ID anemia within 24 h following a single oral administration to reference values. The in vivo investigation demonstrated that the addition of 2 mL of the compound increased the iron content by 14.0 mmol/L. This finding suggests that complex fermented milk products fortified with this compound can effectively be used to prevent iron deficiency and iron deficiency anemia.

Antioxidant activity of fortified milk was assessed, and it was discovered that the incorporation of AsA-Fe-AmA triple chelate complexes led to an overall increase in antioxidant activity. Through sensory evaluation, it was determined that iron ascorbate tryptophanate and iron ascorbate phenylalaninate are ideal in terms of sensory acceptance, suggesting their potential as a potential substance for food fortification in iron deficiency anemia.

Microbiological research revealed that iron ascorbate threoninate, iron ascorbate methioninate, iron ascorbate lysinate, and iron ascorbate tryptophanate have a positive effect on the growth and development of *Lacticaseibacillus rhamnosus* lactic acid cultures at concentrations of 0.0005 mol/L or lower. These findings are significant as they indicate that the complexes not only enhance milk fermentation but also fortify the final product with iron. It was found that fortified fermented milk product has a titratable acidity of 67 ± 1 °T, pH = 4.38 ± 0.05 and
viscosity of 2018 ± 142 Pa·s. Among the AsA-Fe-AmA triple chelate complexes studied, iron ascorbate lysinate emerged as the most effective, demonstrating potential for dietary fortification in iron deficiency anemia.

Acknowledgments

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Limitations

The current study showed promising results of the use of AsA-Fe-AmA triple chelate complexes in fermented dairy products fortification, as well as revealed a gap of full understanding the activity of developed complexes. Thus, in vivo experiment showed the need in 30 days research of rats with modelling ID anemia to understand how AsA-Fe-AmA triple chelate complexes influence on hemoglobin, erythrocytes and hematocrit levels in animals’ blood. Before realization of fortified fermented dairy products, in vivo studies should be carried out with laboratory animals and volunteer humans to assess AsA-Fe-AmA triple chelate complexes for potential toxic effect, digestibility and influence on immunity indicators. Further research should also give understanding of more accurate concentrations of AsA-Fe-AmA triple chelate complexes needed for fermented dairy products fortification. Further sensory evaluation using hedonic test should be carried out with at least 50 untrained panelists to make the results more objective and cleaner.

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