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# Mineral Dialyzability in Milk and Fermented Dairy Products Fortified with FeNaEDTA

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Iron, zinc, and calcium dialyzability and ascorbic acid (AA) concentrations were evaluated in milk and yogurt fortified with FeNaEDTA (FE) or ferrous sulfate (FS) as a control, with or without AA addition. The values obtained for FE iron dialyzability in milk were much higher than those obtained for FS. The addition of AA to milk improved Fe dialyzability when using FS and slightly decreased Fe dialyzability in the FE-fortified nonfermented samples. Milk fermentation increased iron availability from both iron sources. Zinc and calcium dialyzability in products containing any of the two iron sources was increased in fermented milks. EDTA improved Zn dialyzability from intrinsic zinc in every manufactured dairy product. Whereas for milks fortified with FS and stored at 4 °C for 24 h, the AA content remained close to the original concentration, a higher AA degradation was observed when milks were fortified with FE.

**KEYWORDS: Iron; zinc; calcium; mineral availability; ascorbic acid; yogurt; fermentation**

## INTRODUCTION

Because yogurt is a widely accepted dairy food among women, children, and teenagers, it is considered to be a suitable vehicle for fortification with iron (*1*). By using different iron sources at various fortification levels, the effect of yogurt fortification on organoleptic properties has been evaluated (*1, 2*). However, information about mineral bioavailability in this fermented product is scarce. Drago and Valencia (*3*) observed a positive effect of fermentation and lactic acidity on iron and zinc availability from yogurts or acidified milks fortified with ferrous sulfate or ferrous bisglycinate, with or without ascorbic acid. However, no human results showing a possible influence of lactic fermentation on iron and zinc bioavailability have been reported.

When FeNaEDTA (12 mg/L) was used for soy yogurt fortification, a product with good organoleptic properties was obtained; ferrous sulfate addition to yogurts, however, resulted in unpleasant organoleptic properties (*4*).

The International Nutritional Anaemia Consultative Group (*5*) has recently reviewed the use of FeNaEDTA as a food supplement, this compound being suggested as the most suitable iron fortifier in the case of developing countries where programs for the fortification of cereals, legumes, and infant complementary foods are carried out. The advantage of FeNaEDTA lies in the fact that the iron present in this compound is prevented from interacting with phytates, which results in a 2−3 times better absorption, compared to that obtained when other iron compounds are used in fortified diets. Also, because FeNaEDTA has shown no lipid oxidation reactions—which then result in rancidity and the production of oxidized compounds—when added to some cereal-based foods (*5*), it is considered to be a suitable fortifier for mixed diets (*6*). However, studies on the use of FeNaEDTA as a dairy product fortifier or on the interactions between this iron source and intrinsic calcium or zinc are scarce.

On the other hand, it is well-known that cow's milk contains small and variable amounts of ascorbic acid and, so, its oxidation may have little nutritional consequence. However, in the case of iron-fortified dairy products, ascorbic acid is sometimes added to increase iron absorption (*7*). Thermal treatments and incubation processes taking place during yogurt preparation, for example, may accelerate vitamin activity loss. Also, metal ions may catalyze ascorbate oxidation and accelerate ascorbic acid decomposition to dehydroascorbic acid and dicetogulonate (*8*), during both the manufacturing process of yogurt and its later storage; ascorbic acid and iron bioavailability may possibly be affected as a result. It is for this reason that iron bioavailability in fermented fortified milks should be systematically controlled.

The aim of the present work was to study iron, zinc, and calcium availability in milk and fermented dairy products fortified with FeNaEDTA or $FeSO_4$ as a control, with or without added ascorbic acid.

## MATERIALS AND METHODS

**Milk Fortification.** Commercial fluid skimmed milk fortified with 2.5 mg of Fe/100 g was used, fortification being carried out with $FeSO_4$(FS) or FeNaEDTA (FE), with and without the addition of L-ascorbic acid (AA) (31 mg/100 g), which is equivalent to a molar ratio of AA/Fe of 4:1. Before use, these unfermented milks (M-SF,

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**Table 1.** Effect of Fermentation and Ascorbic Acid on the Mineral Dialyzability of Milks Fortified with Ferrous Sulfate (FS) or NaFeEDTA (FE) and on Non-Iron-Fortified (NF) Products[a]

| | % Fe D [b] | | % Zn D [c] | | | % Ca D [d] | | |
|---|---|---|---|---|---|---|---|---|
| sample | FS | FE | FS | FE | NF | FS | FE | NF |
| M | 0.99 ± 0.08a | 35.27 ± 1.55e | 11.32 ± 0.80h | 43.95 ± 2.53j | 7.43 ± 0.74g | 34.54 ± 1.00no | 32.89 ± 0.56n | 34.80 ± 0.85op |
| M-AA | 2.92 ± 0.23b | 30.71 ± 1.63d | 9.74 ± 0.90gh | 47.55 ± 2.06k | 7.96 ± 0.71g | 36.43 ± 0.42pq | 36.49 ± 1.60pq | 35.24 ± 1.3op |
| Y | 4.37 ± 0.27b | 41.73 ± 1.03f | 38.36 ± 2.88i | 59.91 ± 2.25l | 43.51 ± 0.66j | 42.07 ± 1.20r | 42.89 ± 3.09r | 42.17 ± 1.90r |
| Y-AA | 17.42 ± 2.66c | 42.54 ± 0.44f | 45.99 ± 2.04jk | 63.02 ± 0.59m | 46.40 ± 1.83k | 46.31 ± 0.97s | 34.97 ± 1.00op | 42.44 ± 0.68r |

[a] Values are expressed as mean ± standard deviation ($n = 4$). [b] Means for % Fe D having a different letter (a−f) are significantly different ($P < 0.05$). [c] Means for % Zn D having a different letter (g−m) are significantly different ($P < 0.05$). [d] Means for % Ca D having a different letter (n−s) are significantly different ($P < 0.05$).

milk with $FeSO_4$ added; M-SF-AA, milk with $FeSO_4$ and ascorbic acid added; M-FE, milk with FeNaEDTA added; M-FE-AA, milk with FeNaEDTA and ascorbic acid added) were stored during 22 h at 4 °C.

**Preparation of Yogurts.** Stabilized strains of *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* (JOINTEC B13, donated by Diagramma S.A., Argentina), in a 1:1 ratio, were used as lactic bacteria.

The milks with and without fortification were heated at 40 °C and inoculated with dried lactic bacteria previously suspended in milk 1:10; an inoculum of 0.35 mL/L milk was used.

The yogurts used in each treatment were obtained by pouring the milk aliquots into sterilized plastic containers and incubating them at 42 °C until a pH of 4.4 was reached. After the samples (Y-FS, yogurts with $FeSO_4$ added; Y-FS-AA, yogurts with $FeSO_4$ and ascorbic acid added; Y-FE, yogurt with FeNaEDTA added; Y-FE-AA, yogurt with FeNaEDTA and ascorbic acid added) had been cooled in a freezer (−20 °C) during 15 min, they were refrigerated (4 °C) for 16 h, the fermented samples being then analyzed regarding their AA content and their Fe, Zn, and Ca dialyzability.

**Preparation of Control Samples.** To investigate the interaction of the fortification iron with the intrinsic Zn and Ca availability, samples having no iron source addition were used as controls.

**Determination of Lactic Acidity.** Titratable acidity was determined by titration with 0.1 N NaOH using phenolphthalein as indicator and expressed as lactic acid (g/100 g) (AOAC Method 33.2.06) (*9*).

**Determination of Mineral Dialyzability.** The method developed by Miller et al. (*10*), later modified by Wolfgor et al. (*11*), was used. This method, which measures mineral dialyzability under controlled pH conditions after a digestion simulating physiological processes, incorporates buffer PIPES as a mean to obtain a uniform final pH in digest/dialysate systems. To adjust the pH during the digestion and dialysis stage, a PIPES buffer was used, the buffer solution molarity varying according to the matrix to be evaluated. For milks, 0.173–0.181 M, and for yogurts, 0.220–0.237 M, PIPES buffer solutions were used.

**Determination of Ascorbic Acid.** Aliquots of all samples were extracted using 0.85% metaphosphoric acid (MPA) and evaluated by HPLC (Waters Corp., Milford, MA), according to the Behrens and Madére technique (*12*), but using dithiothreitol to reduce the dehydroascorbic acid. As a chromatographic system, a Nucleosil 100-10 C18 reversed-phase column (Macherey-Nagel, Düren, Germany), 25 cm × 4.0 mm i.d., was used; and as mobile phase, a buffer of 80 mM sodium acetate, pH 4.8, containing 15% of methanol (HPLC grade) and 0.015% of MPA was used. The final pH of the mobile phase was 4.6, the flow rate being 0.9 mL/min. Measurements were carried out using a UV detector (Waters model 440) at 254 nm, and the peak areas were measured with a Chromatography Station program (CSW 1.7, DataApex Ltd.).

**Statistical Analysis.** Every experiment was carried out at least twice, and each analysis was performed in duplicate. The results were analyzed by one-way ANOVA. A multiple-comparison procedure of the treatment means was carried out by applying Fisher's least significance difference (LSD) test. Significance of the differences was defined at $p \leq 0.05$.

**Reference Materials.** To validate each batch of analysis for iron, zinc, calcium, and ascorbic acid content, triplicate samples of SRM infant formula 1846 (NIST, Gaithersburg, MD) were run with every set of unknown samples. Agreement of the triplicate mean for the reference material within 5% of the certified value and <5% of relative standard deviation for the triplicates was required for data acceptance.

## RESULTS AND DISCUSSION

**Elaboration of Yogurts.** Approximate fermentation time was 6.6 ± 0.5 h, similar to that obtained in other works (*13*). The initial titratable acidity of yogurts was 0.75 ± 0.03 (g/100 g).

**Comparison of Iron Dialyzability (FeD%) in Milk and Fermented Dairy Products Fortified with Ferrous Sulfate or FeNaEDTA.** The values obtained for FeNaEDTA iron dialyzability in milk were much higher than those obtained for $FeSO_4$ (**Table 1**). Although the absolute values obtained using the in vitro method are higher than those reported in humans, in the latter case, a higher FeNaEDTA iron bioavailability was found in the dairy matrix (13.1 vs 7.9%), compared to that of $FeSO_4$ (*14*). Layrisse and Martinez-Torres (*15*) observed that although milk reduced the absorption of the iron corresponding to ferrous sulfate in 75%, FeNaEDTA absorption was not affected, which would show that the iron forming the complex with EDTA is less sensitive to the inhibitors present in the dairy matrix. Other studies carried out by the same researchers in children and adults showed that the FeNaEDTA is better absorbed than the $FeSO_4$ and $Fe_2(SO_4)_3$ added to milk or maize. Likewise, other studies carried out in preschool children, in which iron-fortified infant formulas based on milk, rice, and sugar were used, showed that the iron present in the FeNaEDTA is 2.6 times better absorbed than that contained in $Fe_2(SO_4)_3$ (*16*). As to the iron dialyzability (% Fe D) data obtained for FeNaEDTA, considerably higher values were reported compared to those obtained for humans, which could be due to the high affinity constant ($pK' = 24$ at pH 6) of the soluble and dialyzable complex (*17*). Although in vivo this fact also contributes to keep iron in solution, it implies that only part of the iron will be bound by the membrane receptor. This process taking place during the absorption is not observed in the in vitro methodology, which in turn results in the high dialyzability percentages found.

High FeNaEDTA availability can result from the fact that the iron is strongly bound in the FeNaEDTA chelate, compared to the iron present in the $FeSO_4$, and this chelate is probably more stable than the iron−casein chelate (*18*). Whereas FeNaEDTA, hemoglobin, and most of the highly chelated iron sources are little affected by other diet components, the bioavailability of noncomplexed iron, as in the case of ferrous sulfate or ferric chloride, depends on the diet composition (*19*).

In the present study, the addition of AA to milk improved % Fe D when using $FeSO_4$, although not in the case of FeNaEDTA, the AA producing a slight decrease of % Fe D in the nonfermented samples in the latter case (**Table 1**).

In a study carried out in humans by MacPhail et al. (*20*), the same bioavailability of the iron corresponding to FeNaEDTA

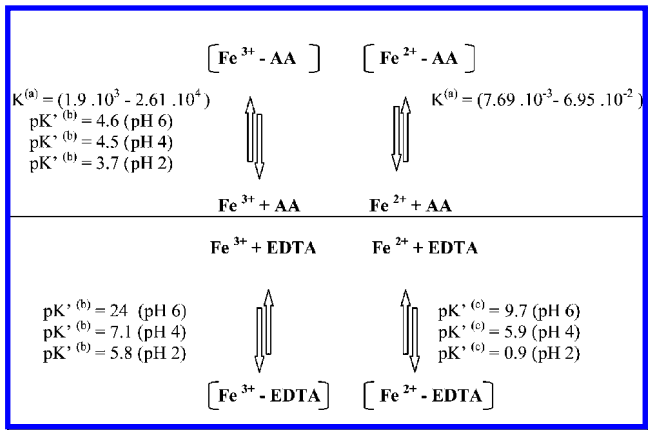


**Figure 1.** Complex formation constant, $K^{(a)}$ (*23*), and apparent complex formation constants, $pK^{(b)}$ (*18*) and $pK^{(c)}$ (*30*), of the ferric and ferrous ions complexed with EDTA and ascorbic acid (AA).

was observed in the case of a maize-based food, with and without AA addition.

As far as vegetables are concerned, Lee and Clydesdale (*21*) reported a decrease in the amount of iron forming the EDTA complex and an increase in the amount of ionic $Fe^{2+}$ after a thermal process in the presence of AA. They concluded that the AA has a significant effect on the physicochemical state of the iron complexed with FeNaEDTA when the food is processed.

The negative effect of AA on iron bioavailability observed in milks, when FeNaEDTA is the fortification source, could be due to EDTA capacity to form complexes, which depends on the pH and the concentration of other metals and ligands present. Accordingly, during the gastric digestion at pH 2, the AA can be competing with the EDTA for the $Fe^{3+}$, which is partly reduced to $Fe^{2+}$, which can in turn interact with casein. This is a comprehensible phenomenon considering that the AA is both a complexing agent and a strongly reducing compound (*22*). From the observation, at acid pH, of the apparent stability constants of the complexes $Fe^{3+}$−EDTA and $Fe^{3+}$−AA (**Figure 1**), a possible AA competition for iron can be inferred. On the other hand, a higher AA/Fe molar ratio—about 4 times in milk and 2 times in yogurt—is found, compared to the EDTA/Fe ratio. Also, AA reducing capacity is especially effective at low pH, at which the potentials of the AA/iron redox system determine the reduction of $Fe^{3+}$ to $Fe^{2+}$ (*22, 23*). The iron reduced during the gastric stage has a lower affinity for the EDTA. The EDTA affinity constant for the $Fe^{2+}$ is approximately 13 times lower than that for the $Fe^{3+}$. Consequently, the AA competition for iron is higher at acid pH, due to the lower EDTA stability constant and the higher AA reduction potential at this pH.

The AA is capable of changing the iron profile in a particular food matrix, and the iron freed from the EDTA and later reduced is more liable to suffer the influence of dietary components. The $Fe^{2+}$ could interact with casein in the case of dairy products and with other food components in complex matrices.

Although in yogurt samples with FeNaEDTA, % Fe D did not decrease with AA addition (**Table 1**), only 48% of the original AA content was observed in the yogurt (**Table 2**). Probably, the high AA destruction that occurred in these samples did not allow corroborating the AA negative effect on the FeNaEDTA present in the fermented products.

The positive effect of fermentation on the % Fe D in samples with FeNaEDTA is not easy to explain. As mentioned above, the stability of the complex EDTA− $Fe^{3+}$ is lower as pH decreases, which would allow other ligands (promoters or inhibitors) to compete for the iron. At pH 4.4, the percentage

**Table 2.** Content of Remaining Ascorbic Acid in Samples Fortified with $FeSO_4$ (FS) or NaFeEDTA (FE) and AA (AA/Fe Molar Ratio = 4:1)

| | remaining AA[a] (%) | | |
|---|---|---|---|
| sample[b] | NF | FS | FE |
| milk | 98.7 ± 2.87a | 96.53 ± 4.35a | 80.69 ± 0.36b |
| yogurt | 94.03 ± 0.93a | 83.33 ± 0.24b | 48.15 ± 3.15c |

[a] Means having a different letters are significantly different ($P < 0.05$). [b] Samples were stored at 4 °C for 22 h after iron fortification.

of the iron bound to EDTA is even higher than that corresponding to other possible competitive ligands (*17*). The free iron fraction could interact with the lactic acid and with the casein and, because the capturing and inhibitory effect of casein for $Fe^{3+}$does not depend on the pH when it is between 4.0 and 6.7 (*24*), the positive union of the $Fe^{3+}$ to the lactic acid is assumed to predominate, which justifies the results here obtained.

**Zinc (% Zn D) and Calcium (% Ca D) Dialyzability in Dairy Fermented Products Fortified with Ferrous Sulfate or FeNaEDTA.** Zinc dialyzability increased 3.4- or 4.7-fold for $FeSO_4$ without or with AA in the fermented milks and 1.3-fold for FeNaEDTA. Calcium dialyzability in products containing any of the two iron sources was increased by about 20% in the fermented milks. Singh et al. (*25*) observed that milk acidification (pH 4.6) led to the solubilization of the calcium and phosphorus contained in the colloidal calcium phosphate and the dissociation of the calcium contained in the casein, with a simultaneous zinc solubilization in the nonmicellar fractions of the milk. Both the hydrolysis of the dairy proteins, which reduces the size of the zinc−casein complexes, and the acidification caused by fermentation, which increases zinc and calcium solubility, could play important roles in the increase of the dialyzability of these minerals.

Although no results regarding the effect of milk fermentation on the bioavailability of the intrinsic zinc in humans have been reported, studies on the calcium bioavailability in fermented products have been published. Smith et al. (*26*) and Recker et al. (*27*) observed no differences in studies carried out in humans as to the absorption of the calcium contained in different dairy products, yogurt among others. The strong homeostatic regulation of calcium absorption and retention could explain these results in studies carried out in humans. Consequently, the small increases found in the calcium dialyzability in fermented or acidified products would have no relevance from a nutritional point of view.

Non-iron-fortified samples, included in this work to investigate the interaction of iron with zinc and calcium (**Table 1**), showed no important changes from a nutritional point of view in the availability of the intrinsic zinc and calcium, compared to the samples fortified with ferrous sulfate.

Higher % Zn D values were obtained for the samples fortified with FeNaEDTA rather than those fortified with ferrous sulfate (**Table 1**). EDTA not only improved % Fe D but also % Zn D in every tested dairy product.

Davidson et al. (*28*) reported an EDTA promoting effect on zinc absorption in humans. Singh et al. (*25*) observed that the addition of 0.2 mM EDTA in skimmed milk caused about 40% of the total zinc to become nonsedimentable by centrifugation. These authors suggested that the EDTA could be making the zinc bound to casein become soluble, because the zinc fraction bound to the colloidal calcium phosphate becomes soluble at higher EDTA concentrations (1–50 mM).

In the present work, sample fortification with FeNaEDTA implied 0.45 mM EDTA, the % Zn D increase produced by the

EDTA being then considered to be due to the solulibilization of zinc bound to casein.

Fermentation increased % Zn D. Both the addition of EDTA and the acid medium increased % Zn D by about 700%, compared with the results obtained for nonfortified milk.

The % Ca D was little affected by the iron source added and by the presence of AA. Singh et al. (*25*) observed that the calcium nonsedimentable fraction showed no variation with EDTA addition (0.1–1.0 mM), which indicates that the colloidal calcium phosphate and the casein micelles suffer no modifications at the EDTA concentrations removing 40% of the micellar zinc. Using the double-isotopic technique, Davidson et al. (*28*), on the other hand, reported FeNaEDTA having no effect on the calcium fractional absorption in humans.

The acid medium positive effect observed on the % Ca D, when using ferrous sulfate, was not verified when using FeNaEDTA, the conclusion once more being that the small modification observed in the calcium dialyzability of iron-fortified and fermented products would have no relevance from a nutritional point of view.

It is important to point out that, although EDTA has been selected as an example of ferric chelate, its selection as a dairy product fortifier should be considered with caution, because this type of food is used for children and its ingestion in high amounts could exceed the EDTA acceptable daily intake (2.5 mg/kg by weight) (*5*).

Also, this compound is not recognized as an iron fortificant by Argentine legislation, its use being recommended (*5*) in programs of food fortification in developing countries, and the U.S. FDA recently considered it as a GRAS (Generally Recognized As Safe) food for its use as a fortificant in soy, fish, teriyaki, and hoisin sauces at a level of 0.024% iron by weight and in sweet and sour sauce at a level of 0.012% iron by weight. Akzo Nobel also reports that Kraft Foods Global submitted a GRAS notice (GRN 000152) for the use of FeNaEDTA as an iron fortificant in powdered meal replacement, flavored milk, and fruit-flavored beverages.

**Effect of the Addition of Different Iron Sources on the Ascorbic Acid Content in Dairy Products.** An iron pro-oxidant tendency on the AA was observed in every manufactured product. Although for milks fortified with FS and stored at 4 °C for 24 h the AA content remained close to the original concentration, a higher AA degradation was observed when milks were fortified with FeNaEDTA (**Table 2**). A significantly higher AA degradation was observed when fermented milks were fortified with FeNaEDTA rather than with FS.

The AA remaining content was lower in every sample fortified with NaFeEDTA than in those to which the other iron source had been added. This shows a stronger pro-oxidant effect of the iron corresponding to EDTA compared to that of ferrous sulfate. Iron capacity to form complexes with soluble chelating agents, such as EDTA and citrate, does not prevent it from taking part in the Haber−Weiss cycle. This cycle can be started by many reducing substances such as AA, which would represent a continuous $Fe^{2+}$ source. Both the availability for redox reactions of the $Fe^{3+}$ chelated by the EDTA and the high solubility of the complex EDTA−$Fe^{3+}$ would make this complex a very efficient oxidation catalyst in these matrices, although it is generally used to prevent oxidative rancidity (*29*).

These results show that the pro-oxidant effect of the different iron sources depends on the food matrix, its pH, and the presence of antioxidants. Such an effect could affect the oxidation of the ascorbic acid added and of the lipids and vitamins present in these products.

It is necessary to point out that industrial processes to obtain yogurt differ from those used by us at laboratory level, because the former involve the addition of other components (sugar, milk powder, stabilizers, etc.), homogenization, and pasteurization of this mix before ferment inoculation. In such conditions, AA degradation would be higher than that obtained in this study.

In the case of milks fortified with FeNaEDTA, the already high % Fe D and % Zn D are significantly increased by fermentation. The % Ca D was not affected by the presence of FeNaEDTA, at the concentration used for fortification. However, the addition of AA had a negative effect in % Fe D.

Fermented dairy products have the advantage of increasing the availability of not only Fe but also Zn. Although it would be necessary to carry out in vivo studies to confirm our in vitro results, it is evident that the correct formulation of iron-fortified yogurts would depend on the iron source selected.

Because the fortification with iron can accelerate AA degradation in the stages before and during fermentation, and also during the product shelf life, the pro-oxidant effect of this iron should be taken into account at the formulation step.

Although there are numerous food grade iron sources, it is necessary to select the most adequate according to its reactivity and bioavailability in the food to be fortified. Besides, the use of mineral absorption promoters must be selected according to the food matrix and iron source utilized. These results may serve to guide food technologistsm but bioavailability studies in humans are warranted.

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