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Ferritin synthesis by Caco-2 cells as an indicator of iron bioavailability: Application to milk-based infant formulas

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Abstract

The bioavailability of iron from milk-based infant formulas was estimated by an *in vitro* system including enzymatic digestion, iron uptake by Caco-2 cells and ferritin determination via an enzymoimmunoassay (ELISA). Positive correlations ($p < 0.01$) were found between the Fe(II) added to Caco-2 cells and ferritin synthesis and between the amount of dialyzed iron added to the cell culture and ferritin synthesis. The comparison of the bioavailability of iron from different milk-based formulas showed that adapted formulas having the same composition but differing in the iron salts added yielded similar ferritin levels. The same happened with follow-up formulas differing only in the presence or absence of bifidobacterium added. However, significant differences in the amount of ferritin synthesized were recorded between the two analyzed toddler formulas. Such differences could be attributed firstly to the ascorbic acid content and perhaps also to the manufacturing process involved, because one formula was in liquid form while the other was powdered.

Keywords: Ferritin; Caco-2 cells; Iron bioavailability; *In vitro* digestion; Infant formulas

1. Introduction

Dietary deficiency of iron and the resulting anemia is one of the most prevalent nutritional disorders in infants. The fortification of infant formulas with iron is one of the strategies adopted to prevent this deficiency. Iron content and bioavailability (BA) determine the value of an infant formula as a dietetic source of iron. *In vivo* and *in vitro* studies are used to estimate mineral BA. *In vitro* methods comprise the enzymatic gastrointestinal digestion of food and an estimation of the element in soluble form and/or dialyzable through a dialysis membrane of a certain pore size (Wienk, Marx, & Beynen, 1999). The incorporation of a Caco-2 cell culture to these models has improved the methodology. Caco-2 cells in culture differentiate spontaneously, both structurally and functionally, into cells resembling mature enterocytes (Hidalgo, Raub, & Borchardt, 1989; Pinto et al., 1983). The low cost, ease of use and widespread acceptance of the Caco-2 cell line make this model an attractive alternative to animal studies and a valuable tool for use in conjunction with human trials.

Studies on the uptake of iron from infant formulas using Caco-2 cells are scarce. In these studies iron uptake has been estimated by measuring intracellular iron using radioisotopes (Glahn, Olivia, Yeung, Goldman, & Miller, 1998) and atomic absorption spectrophotometry (Jovaní, Barberá, Farré, & Martin de Aguilera, 2001, 2004), and by determining intracellular ferritin with an immunoradiometric assay (Etcheverry, Wissler, Wortley, & Glahn, 2004). Ferritin determination by ELISA has only been applied to model systems (Álvarez-Hernández, Nichols, & Glass, 1991; Garcia-Casal, Leets, & Layrisse, 2004; Tapia, Arredondo, & Núñez, 1996). The use of ferritin synthesis as an indicator of iron uptake by Caco-2 cells affords a highly sensitive and accurate measure of the availability of iron from foods, and eliminates the need for extrinsic or intrinsic labeling of food iron in availability assays (Glahn et al., 1998).

An *in vitro* study has been carried out using a system that includes enzymatic digestion, iron uptake by Caco-2

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cells, and measurement of the ferritin formed via enzymo-immunoassay (ELISA), with the aim of detecting possible differences in the bioavailability of iron from different types of infant formulas. Previously, the appropriate culture conditions for Caco-2 cells and the relationship between added iron and ferritin synthesis were established.

2. Materials and methods

2.1. Chemicals and enzymes

Enzymes and bile salts were purchased from Sigma Chemical Co. (St Louis, MO, USA): pepsin (Porcine: cat no. P-7000, enzymatic activity 944 U/mg protein), pancreatin (Porcine: cat. no. P-1750, activity equivalent to $4 \times$ US Pharmacopoeia specifications/mg pancreatin), and bile extract (Porcine: cat. no. B-8631, glycine and taurine conjugates of hyodeoxycholic and other bile salts).

Buffer (blank), pH 7, contained 130 mM NaCl, 10 mM KCl, 1 mM $MgSO_4$, 50 mM HEPES and 5 mM glucose. Stock solution, freshly prepared before each experiment, contained aqueous 10 mM $FeCl_3 \cdot 6H_2O$ and 20 mM nitrilotriacetic acid (NTA) or 2.5 mM $FeSO_4 \cdot 7H_2O$ and 13 mM ascorbic acid in metaphosphoric acid-acetic acid solution (15 g $HPO_3 + 40$ ml CH_3–COOH to 500 ml in H_2O).

Caco-2 cell ferritin was measured with an enzyme immunoassay marketed for the quantitative determination of ferritin in human serum. The assay system uses an antihuman rabbit ferritin. The kit used was purchased from Ramco Laboratories (Spectro Ferritin, Catalog number S-22, Ramco Laboratories Inc., Stafford, TX, USA).

All reagents used were of reagent grade, and distilled deionized water (DDW; Millipore Ibérica S.A., Barcelona, Spain) was used throughout the experiments.

2.2. Samples

Milk-based adapted infant formulas with added ferrous sulphate (A^S) or ferrous lactate (A^L), and follow-up (F) infant formulas and toddler (T) formulas with (FB, TB) or without (F, T) *Bifidobacterium bifidum* and *Bifidobacterium longum* added with ferrous sulphate were studied. The composition of the samples is reported in Table 1.

2.3. In vitro digestion: dialysis method

The method of Jovaní, Viadel, Laparra, Barberá, and Farré (2004) was applied. Millipore-Milli Q distilled deionized water (≈80 ml) was added to the different sample weights (10 g A^S and A^L, 7 g F and FB, 40 g T and 5 g TB) in order to obtain similar iron contents. The pH was adjusted to 2.0 with 6 N HCl, checked after 15 min, and if necessary readjusted to 2.0, and a sufficient amount of freshly prepared pepsin (previously demineralized) was added to yield 0.02 g/sample. The sample was made up to 100 g with distilled deionized water and incubated at 37 °C for 2 h in a shaking water bath (Model SS40-2, Gran Instrument). The gastric digest was maintained in ice for 10 min.

Table 1
Composition of the analyzed samples referred to 100 ml ready to eat sample (manufacturer supplied data)

	$A^{S,L}$	F, FB	T	TB
Energy (kcal)	68	65	65	61
Protein (g)	1.5	2.0	2.6	1.9
Casein (g)	0.7	1.0	1.7	1.2
Fat (g)	3.8	3.3	2.5	2.6
Carbohydrates (g)	7.1	7.0	8.0	7.7
Lactose (g)	7.1	3.5	8.0	2.5
Maltodextrins (g)	0	3.5	0	5.3
Ca (mg)	51	78	100.0	110.5
Fe (mg)	0.8	1.3	1.3	1.2
Zn (mg)	0.6	0.5	0.8	0.6
Ascorbic acid	6.8	6.4	6.0	12.4
Ascorbic acid/Fe molar ratios	2.7	1.6	1.5	3.3

A, adapted formula (supplemented with ferrous sulphate (A^S) or ferrous lactate (A^L)); F, follow-up formula; FB, follow-up formula with *bifidobacterium*; T, toddler formula; TB, toddler formula with *bifidobacterium*. $A^{S,L}$, F, FB and TB were powdered samples and were reconstituted according to manufacturer instructions (13% w/v).

A dialysis bag (molecular mass cut-off of 10,000–12,000 kDa) containing 25 ml water and an amount of $NaHCO_3$ equivalent to the titrable acidity (as previously measured) was placed in the flasks together with 20-g aliquots of the pepsin digest. Incubation was continued for 30 min, and a sufficient amount of freshly prepared pancreatin–bile salts solution (previously demineralized) was added to yield 0.001 g and 0.006 g of pancreatin and bile salts per 20-g aliquots. Incubation was then continued up to 2 h. Finally, the dialysis bag (dialysate content) was removed and used in the Caco-2 cell uptake assays.

2.4. Cell culture

Caco-2 cells were obtained from the European Collection of Cell Cultures (ECACC 86010202, Salisbury, UK), and were used between passages 56 and 95. The cells were seeded at a density of 50,000 cells/cm^2 in 6-well plates and were maintained at 37 °C in an incubator (NH-4500E Model Nuaire, Minnesota, USA) under a 5% CO_2/95% air atmosphere at constant humidity. The cells were grown in minimum essential medium (MEM; Gibco BRL Life Technologies, Scotland) with 10% v/v fetal bovine serum (FBS), 1% v/v non-essential amino acids (Gibco), 1% v/v L-glutamine (Biowhittaker), 1% v/v antibiotic solution (penicillin–streptomycin) (Biowhittaker) and 0.1% v/v fungizone (Gibco) at pH 7.2–7.4. This MEM is designated as MEM–FBS. The basal amount of iron in the MEM–FBS was 7.03 μM. To grow cells under low iron conditions, MEM supplemented with FBS depleted of iron, obtained by titrating the FBS at pH 4.5 in the presence of 300 g/l Chelex (Álvarez-Hernández et al., 1991). The iron-free MEM is designated as MEM–FBS–FeF (iron content 1.16 μM). Culture medium was changed every 2 days.

The iron uptake assays were performed with differentiated cells 14–16 days after seeding.

At the end of each assay, the cell monolayers were washed three times with buffer solution, and the cells were detached with trypsin–EDTA solution. These cells were used in intracellular iron measurement.

2.5. Ferritin measurements: experimental design

Three series of assays were carried out using standard solutions or infant formulas.

Assay 1. The aim was to evaluate the effect on ferritin synthesis of using MEM–FBS or MEM–FBS–FeF in the culture media. To this effect three culture conditions were used: cells seeded and grown in either, MEM–FBS, MEM–FBS–FeF and MEM–FBS–FeF added with 7.03 μM Fe (iron content in MEM–FBS).

Assay 2. The aim was to assess the relationship between the added iron (10 or 65 μM Fe(III)) and the amount of ferritin synthesized, and between intracellular iron and ferritin synthesized in: (a) cells grown in MEM–FBS with the addition of 10 and 65 μM Fe(III) and (b) in cells grown in MEM–FBS–FeF with the addition of: (1) 5, 10 and 65 μM of Fe(III); and (2) 650 μM ascorbic acid and 1, 2, 5 and 10 μM Fe(II) (see Table 1).

Assay 3. The aim was to assess the bioavailability of iron from milk-based infant and toddler formulas, using as criteria the amount of ferritin synthesized, and to detect differences among them.

In assays 2 and 3, immediately before addition of the standard solution or dialyzed fraction, respectively, the growth medium was removed from each well, and the cell monolayer was washed three times with MEM at 37 °C. Then, the standard solution in MEM–FBS–FeF (2 ml) or 1 ml of the dialysate plus 1 ml of MEM–FBS–FeF was pipetted into one culture well, and the plate was incubated for 22 h at 37 °C in 5% CO_2 with 95% relative humidity.

2.6. Analytical determinations

2.6.1. Iron determination

The iron contents of blank, standard solutions, infant formulas, dialyzed fractions and cell monolayer (intracellular iron) were determined by atomic absorption spectrophotometry (AAS) (model 2380; Perkin–Elmer, Norwalk, CT, USA). Prior to iron determination, the organic matter of the infant formulas and cell monolayer was destroyed by ashing at 450 °C (model K1253A; Heraeus instruments, Hanau, Germany).

2.6.2. Ferritin determination

At the end of each assay, the cell monolayers were washed three times with buffer solution, and the cells were detached with trypsin–EDTA solution. Cell monolayers were collected with 2 ml of deionized water at 4 °C and homogenized at 17,000 rpm for 3 min at 4 °C (Polytron® PT 2000. Kinematica AG). Ten-microliter aliquots of the sonicated Caco-2 monolayer were used in ferritin measurement.

2.6.3. Protein determination

Cell protein content was determined according to Lowry, Rosebrough, Farr, and Randall (1951), for expressing the ferritin contents as ng ferritin/mg protein, and to evaluate reproducibility of the cultures.

2.7. Statistical analysis

The results obtained were analyzed by one-way analysis of variance (ANOVA). Selected pairs of means were compared by Tukey's test. Values of $p < 0.05$ were considered significant. A simple regression analysis was also applied with the aim of estimating the possible relationship between ferritin synthesis and iron addition or cell content. The Statgraphics Plus version 5.0 statistical package (Rockville, Maryland, USA) was used throughout.

3. Results and discussion

3.1. General

As reported in the experimental design, preliminary assays were carried out to select the culture media suited for the ferritin synthesis assay (assay 1), and to evaluate the relationship between iron addition to Caco-2 cells and ferritin synthesis (assay 2).

3.2. Assay 1

Iron uptake and ferritin synthesis depend on the iron status of the cell. Ferritin synthesis was lower in cells grown in MEM–FBS–FeF (1.46 μM Fe) than in MEM–FBS (7.03 μM Fe), 3.58 ± 1.26 versus 7.51 ± 0.48 ng ferritin/mg protein. The use of MEM–FBS–FeF added with an amount of Fe similar to that of MEM–FBS (7.03 μM) increased the synthesis of ferritin 23-fold (82.64 ± 10.64 versus 3.58 ± 1.26 ng ferritin/mg protein). This suggests that the iron in FBS is less available to the cells than the added inorganic iron, as previously reported by Gangloff et al. (1996).

It is clear that the synthesis of ferritin by Caco-2 cells depends on the amount and availability of iron in the culture medium, as reported by different authors (Álvarez-Hernández et al., 1991; Gárate & Núñez, 2000; Núñez, Tapia, Toyokuni, & Okada, 2001), and on the iron status of the cells (Tapia et al., 1996).

3.3. Assay 2

Ferritin contents obtained by different iron additions to cells grown in MEM–FBS, or in MEM–FBS–FeF, are reported in Table 2. After adding Fe^{3+}-NTA, an increase in ferritin synthesis in accordance with the amount of iron added was observed. The increase was higher in cells grown

Table 2
Ferritin synthesis resulting from the addition to Caco-2 cells of standard solutions of iron

	Fe added (μM)	Ferritin/protein (ng/mg)
MEM–FBS		
Fe(III)	0	7.51 ± 0.48
	10 (1.12)	13.1 ± 5.94
	65 (7.28)	71.8 ± 3.63
MEM–FBS–FeF		
Fe(III)	0	4.67 ± 0.95
	5 (0.56)	11.6 ± 3.86
	10 (1.12)	29.0 ± 3.37
	65 (7.28)	307 ± 14.5
Fe(II) + ascorbic acid (650 μM)	0	9.66 ± 1.57
	1 (0.11)	9.59 ± 1.33
	2 (0.22)	11.4 ± 2.55
	5 (0.56)	72.1 ± 12.4
	10 (1.12)	108 ± 13.2

MEM–FBS, culture medium; MEM–FBS–IF, culture medium iron free. Values in brackets correspond to the amount (μg of iron) added in the assay.

in MEM–FBS–FeF than in MEM–FBS. In the first case, a significant ($p < 0.01$) linear correlation was found between the Fe(III) added and ferritin synthesis (ferritin formation $= -8.68579 + 4.83087 \times$ Fe added, $R^2 = 99.30\%$). The results corroborate that the initial iron status of the cells determines iron uptake estimated by ferritin synthesis, and consequently the following studies were carried out in cells grown in MEM–FBS–FeF.

The simultaneous addition of Fe(II) and ascorbic acid yielded greater ferritin synthesis than the addition of only Fe(III) (see Table 2). This can be explained by a greater uptake and therefore greater ferritin synthesis by Caco-2 cells exposed to Fe(II) when compared to Fe(III). Caco-2 cells more rapidly take up and transport Fe(II) from Fe(II)-ascorbate than Fe(III) from compounds such as Fe^{3+}-NTA (Álvarez-Hernández et al., 1991, Alvarez-Hernandez & Smith, 1994), and that non-heme iron must be converted to Fe(II) either by endogenous or dietary reductants (such as ascorbic acid) or by ferrireductase activity on the brush border membrane surface of Caco-2 cells prior to uptake by cells (Han, Failla, Hill, Morris, & Smith, 1995).

Positive linear correlations ($p < 0.01$) were found between Fe(II) (ascorbic acid) added and ferritin contents (ng/mg protein) (ferritin $= 2.669 + 10.99 \times$ added Fe, $R^2 = 94.36\%$); and also between intracellular Fe contents (μg/mg cell protein) and ferritin contents (ng/mg protein) in Caco-2 cells (ferritin $= -91.408 + 125.099$ Fe intracellular content, $R^2 = 91.83\%$).

The advantages of measuring ferritin contents instead of intracellular iron in Caco-2 cells by AAS are the simplicity of the procedure, because it does not require the tedious sample treatment (including organic matter destruction) applied in AAS, and the prevention of possible errors due to iron bound to the cell surface and not truly taken up by the cells.

3.4. Assay 3

The obtained total and dialysate iron contents, expressed as μg iron/g of formula, are reported in Table 3. Ferritin contents in Caco-2 cells of iron dialyzed from the formulas expressed as ng ferritin/mg cell protein, and (ng ferritin/mg cell protein/μg added iron), are reported in Table 4.

In uptake assays with Caco-2 cells, dialysates had iron contents ranging from 0.14 to 0.55 μg (Table 3). In contrast to the positive linear correlations obtained between ferritin contents and added iron from standards, the correlation between added dialyzed iron and ferritin contents was: ferritin $= 1/(0.011 + 0.005/$dialyzed iron added; $R^2 = 59.68\%$).

Adapted formulas had the same composition except for the iron salt (sulphate A^S or lactate A^L), which did not affect either dialyzed iron (Table 3) or ferritin synthesis (Table 4). This observation agrees with the lack of significant differences in the dialysability of iron from infant formulas added with different iron salts (Domínguez et al., 2004), and also with the fact that in rats iron absorption depends more on the dose used than on the type of iron salt involved (Yun-Ji, 1999).

Follow-up formulas had the same composition except for the inclusion of *B. bifidum* and *B. longum* in FB, that did not affect either the dialyzed iron (Table 3) or ferritin synthesis (Table 4).

The two toddler formulas differ in their composition, the addition or not of *Bifidobacterium*, and the presentation form or physical state; one is liquid (T) and the other powdered (TB). Although both formulas had similar iron contents, the iron dialysate from TB was about half that from T (Table 3), while ferritin synthesis resulting from the addition of the dialysate was much higher in TB than in T (Table 4).

As a consequence of the thermal treatment applied during the manufacturing process, liquid infant formulas have a higher hydrolyzed protein content than powdered formu-

Table 3
Total (μg/g formula) and dialyzed (μg/g formula) iron

Formulas	Total content (μg/g IF)	Dialyzed (μg/g IF)
Adapted		
A^S	45.9 ± 4.50	2.50 ± 0.80
A^L	47.5 ± 4.30	2.30 ± 0.60
Follow-up		
F	90.1 ± 3.70	6.40 ± 1.10
FB	90.1 ± 5.50	6.30 ± 0.90
Toddler		
T[a]	78.0 ± 2.8	12.2 ± 1.4
TB	73.0 ± 1.90	6.70 ± 0.80

Adapted infant formula: A^S, ferrous sulphate added; A^L, ferrous lactate added.
Follow-up infant formula: F, without *bifidus* added; FB, with *bifidus* added.
Toddler formula: T, without *bifidus* added; TB, with *bifidus* added.
[a] Liquid sample (contents are referred to dry product).

Table 4
Absolute (ng/mg cell protein) and fractional (ng/mg cell protein/μg Fe added) Caco-2 cell ferritin levels from milk-based formulas and iron (μg) provided by the dialysate

Infant formulas	Fe provided by the dialysate (μg)	Ferritin	
		ng/mg cell protein	ng/mg cell protein/μg Fe added
Adapted			
A^S	0.14 ± 0.03	17.8 ± 2.82	127 ± 20.1[a,b]
A^L	0.14 ± 0.03	22.8 ± 4.19	163 ± 29.9[a]
Follow-up			
F	0.40 ± 0.06	36.2 ± 6.82	90.5 ± 17.1[b,c]
FB	0.38 ± 0.06	45.0 ± 7.17	118 ± 18.9[a,b]
Toddler			
T	0.55 ± 0.05	34.5 ± 8.09	62.7 ± 14.7[c]
TB	0.27 ± 0.05	83.1 ± 7.22	308 ± 86.7[d]

Adapted infant formula: A^S, ferrous sulphate added; A^L, ferrous lactate added.
Follow-up infant formula: F, without *bifidus* added; FB, with *bifidus* added.
Toddler formula: T, without *bifidus* added, liquid form; TB, with *bifidus* added.
Basal ferritin contents 3.58–6.06 ng ferritin/mg cell protein.
Non-coincidence of superscripts (a, b, c, d) denotes statistically significant differences ($p < 0.05$) between values in the same column.

las, and this could be responsible for the higher iron dialysis found in liquid versus powdered formulas (Sarriá & Vaquero, 2001). Hydrolysis may reduce the number of available iron ligands and be responsible for the increase in iron dialysis, as found in a previous study where the highest iron dialysis value corresponded to IF with hydrolyzed proteins (García, Alegría, Barberá, Farré, & Lagarda, 1998). All this could justify the higher iron dialysis of the liquid toddler formula when compared with the powdered formula (see Table 2).

Despite the fact that the addition to Caco-2 cells of iron from TB was half that from T, higher ferritin contents were obtained with TB (Table 4). The difference cannot be ascribed to the presence of *Bifidobacterium*, because no significant differences were found between the F and FB follow-up formulas. However, it could be due to differences in ascorbic acid content, in ascorbic acid/iron molar ratio, and in the manufacturing processes applied to obtain the formulas.

Both ascorbic acid content and ascorbic acid/iron molar ratio were higher in TB than in T (Table 1). In fact, of all the studied formulas, the highest ascorbic acid/iron molar ratio corresponded to TB, i.e., those yielding the highest ferritin contents. This can be explained by the enhancer effect of ascorbic acid in the uptake of iron from standard solutions, as previously reported, and from infant formulas (Etcheverry et al., 2004; Han et al., 1995). The enhancer effect of ascorbic acid primarily seems to be due to reduction rather than chelation of Fe (III), suggesting that Fe(II) is the species efficiently transported across the brush border membrane (Han et al., 1995).

The physical state (T being liquid and TB powdered/solid) could also affect iron bioavailability. Ultrahigh temperature-sterilized infant formulae, and in particular bottle-sterilized infant formulae, have a higher lactulose content, a higher lactulose to furosine ratio (two thermal treatment markers), and greater protein denaturalization than powered formulas. In addition, bottle-sterilized formulas could contain iron bound to advanced Maillard reaction products (MRPs) or melanoidins – giving rise to non-absorbable iron compounds. In rat pups fed with liquid formulas a decrease in iron bioavailability – possibly as a consequence of the presence of MRPs, altered proteins and lactulose was found (Sarriá & Vaquero, 2001, 2004). A hypothesis is that MRPs and altered proteins resulting from the thermal treatment could justify the low ferritin synthesis observed in the toddler liquid formula when compared with the powdered formula.

Iron contents (μg/g of infant formula) and the ng ferritin synthesized/mg cell protein/g sample are represented in Fig. 1. The lack of a linear correlation between iron content in the formulas and ferritin contents stands out, confirming the effect of sample composition (mainly as regards ascorbic acid content) and the manufacturing process upon iron uptake and ferritin synthesis, and therefore on iron bioavailability.

In the present study, Caco-2 ferritin levels ranging from 18 to 83 ng ferritin/mg cell protein were obtained, the lowest value corresponding to the formulae with the lowest iron content (8 mg iron/l, A^S, A^L), and the highest to the toddler formula with *Bifidus* added (TB) – the latter containing the highest ascorbic acid content, as previously reported. The low ferritin levels obtained in our study, when compared to those reported by Etcheverry et al. (2004), can be mainly explained by differences in formula composition. There were also differences in the method used to measure ferritin (ELISA versus immunoradiometric). For all these reasons, it is not possible to compare absolute values; rather, formulas are ranked according to iron bioavailability.

In summary, in adapted and follow-up milk-based infant formulae, the type of iron salt added (sulphate or lactate) to the former, or the presence of bifidobacteria in the latter, did not affect either the amount of dialyzed iron

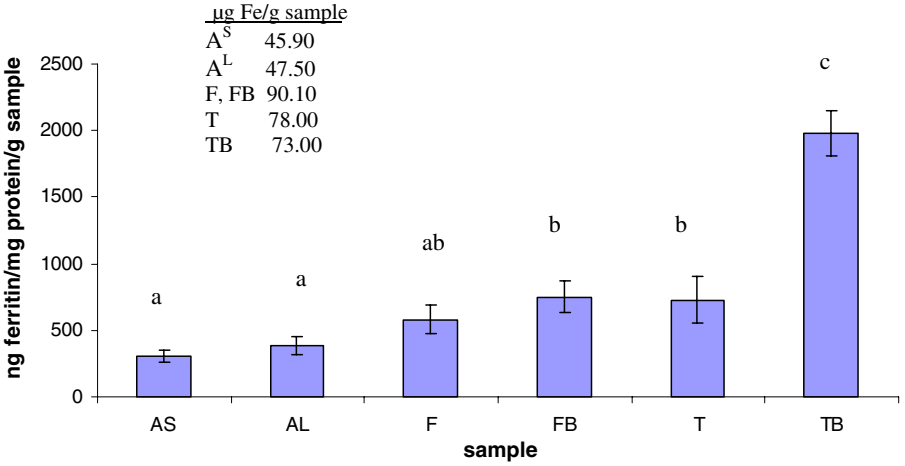

Fig. 1. Caco-2 ferritin formation per gram of infant formula. Adapted infant formula: AS, ferrous sulphate added; AL, ferrous lactate added. Follow-up infant formula: F-without *bifidus* added; FB, with *bifidus* added. Toddler formula: T, without *bifidus* added, liquid form; TB, with *bifidus* added. Non-coincidence of superscripts (a–d) denotes statistically significant differences ($p < 0.05$) between values in the same column.

or the amount of ferritin synthesized. The significant difference ($p < 0.01$) found between the two toddler formulas (T and TB) in terms of dialyzed iron and ferritin synthesis is mainly attributable to differences in ascorbic acid content and perhaps also to the presence of Maillard reaction products resulting from the thermal treatment applied during the manufacturing process, although this is only a hypothesis.

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