Supplemental inulin does not enhance iron bioavailability to Caco-2 cells from milk- or soy-based, probiotic-containing, yogurts but incubation at 37 °C does

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Abstract

The *in vitro* effects of supplemental inulin (4%) on iron (Fe) availability in two different probiotic-containing yogurts were examined. Milk or soy-based yogurts, with and without inulin, were incubated (37 °C) for 48 h or without any incubation before comparison by an *in vitro* gastrointestinal digestion/Caco-2 cell culture model was used to assess iron bioavailability. The dialysable Fe fraction, cell ferritin formation, and cell associated Fe were monitored. Supplemental inulin decreased dialysable Fe only in non-incubated milk-based yogurt. In both yogurts incubation by itself increased dialysable Fe, and inulin increased the latter only in soy-based yogurt. Cellular ferritin concentration were higher after exposure to non-incubated milk-based than soy-based yogurt, although, after incubation the latter induced the highest ferritin formation. These data suggest that inulin does not have a direct effect on Fe bioavailability in the small intestine, and that probiotic bacteria play an enhancing role on Fe bioavailability.
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1. Introduction

Iron (Fe) deficiency is a leading nutritional concern worldwide, affecting 20–50% of the world's population (Beard & Stoltzfus, 2001). Common prebiotics such as inulin have been hypothesized to have an enhancing effect on iron absorption (Greger, 1999; Yeung, Glahn, Welch, & Miller, 2005). However, results from studies related to the effects of inulin on Fe availability are variable (Bosscher, Van Caillie-Bertrand, Van Cauwenbergh, & Deelstra, 2003; Van den Heuvel, Schaafsma, Muys, & van Dokkum, 1998; Yasuda, Ronecker, Miller, Welch, & Lei, 2006). In an *in vitro* study, Bosscher et al. (2003) showed a decreased Fe dialysability from infant formula when supplemental inulin (3%) was added. In contrast, adding 4% inulin to a corn/soybean diet resulted in increased Fe absorption in young pigs (Yasuda et al., 2006). In young healthy men, indigestible fructooligosaccharides did not affect calcium and nonhaem-iron absorption (Van den Heuvel et al., 1998). Several other studies using *in vivo* models showed that inulin and other indigestible oligosaccharides enhanced Ca, Mg, Cu, and Zn absorption in rats (Coudray, Demigné, & Rayssiguier, 2003; Coudray, Feillet-Coudray, Gueux, Mazur, & Rayssiguier, 2006; Coudray, Feillet-Coudray et al., 2005; Coudray et al., 2005).

Yogurt is one of the best known food products that contains probiotics and increasingly yogurts are being supplemented with prebiotics by manufacturers. In addition, yogurt exhibits some characteristics that have the potential to decrease the inhibitory effect of other dietary compounds on micronutrient bioavailability (i.e., the low

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pH of yogurt may reduce the inhibitory effect of dietary phytic acid on mineral bioavailability because an acidic environment may lower the affinity of phytic acid for mineral cations) (Adolfosson et al., 2004). Rosado, Díaz, González, Griffin, and Abrams (2005) showed that addition of milk-based yogurt to a plant-based meal significantly increased Zn absorption but did not affect Fe absorption. Yeung et al. (2005) suggested that the fermentation of prebiotics by natural microflora present in the colon may enhance Fe uptake by different mechanisms. However, one question that has not been addressed in published studies is: Do prebiotics have to reach the colon before they exert an enhancing effect on Fe uptake (Yeung et al., 2005)?

Presumably, soluble dietary fibre has the capacity to bind mineral cations (reviewed in Coudray et al., 2003). These effects could be expected to negatively influence mineral bioavailability in the duodenum. Bosscher et al. (2003) reported lower Fe dialysability from infant formulas supplemented with 3% inulin using an *in vitro* continuous flow dialysis model. However, supplemental inulin increased soluble iron concentrations in the proximal, mid, and distal colon of pigs (Yasuda et al., 2006). Thus, it appears that the effects of prebiotics are poorly understood.

The Caco-2 cell line is a human adenocarcinoma cell line that has proven to be a useful model for studying Fe absorption (Glahn, Lee, Yeung, Goldman, & Miller, 1998; Zhu, Glahn, Yeung, & Miller, 2006). Under appropriate conditions, they develop microvilli and in many ways act similarly to small intestinal epithelial cells (Pinto et al., 1983). These characteristics, among others, make this model system an attractive alternative to animal models.

In view of growing interest in functional foods, and the scarce and variable data regarding the effects of prebiotics such as inulin on Fe uptake, this study was designed to compare Fe bioavailabilities from two different probiotic-containing yogurts, one milk-based and the other soy-based, when supplemental inulin was added. An *in vitro* gastrointestinal digestion/Caco-2 cell culture model was used to assess bioavailability.

2. Material and methods

2.1. Reagents

Unless otherwise stated, all reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All glassware used in sample preparation and analyses was rinsed with 10% (v/v) concentrated HCl (37%) and 18 MΩ deionized water before use in order to avoid mineral contamination.

2.2. Instruments

Total Fe content was determined with an inductively coupled argon plasma emission spectrometer (ICP-ES,

Model 61E Trace Analyzer, Thermo Jarrell Ash Corporation, Franklin, MA, USA) after wet-ashing. Other equipment used included a spectrophotometer (DU 520 UV/vis, Beckman Coulter, Palo Alto, CA, USA), and an automatic gamma counter Wizard 3 Wallac 1480 (Perkin Elmer, Norwalk, CT, USA).

2.3. Samples

Two different, commercially available yogurts, milk- (MB) and soy-based (SB), were purchased in food stores in Ithaca, New York (USA). Both samples contained probiotic live cultures, as indicated by the manufacturer; sample MB (milk-based) contained *L. bulgaricus*, *S. thermophilus*, and *Bifidobacterium* sp., and sample SB (soy-based) contained *L. bulgaricus*, *L. acidophilus*, *L. casei*, *L. rhamnosus*, *S. thermophilus*, *B. bifidum*. The moisture content of the samples were; MB 78.3% and SB 80.0%.

To evaluate the effect of extrinsic inulin, aliquots (1.06 ± 0.03 g) of fresh samples were transferred to 50 mL centrifuge tubes and 5 mL of an isotonic saline solution [140 mM NaCl, 5 mM KCl] was added. Afterwards, inulin (0.041 ± 0.001 g) was mixed into the samples and the mixtures were divided in two sets. One of the prepared sets was subjected to *in vitro* digestion once prepared, and the other after incubation (37 °C/5% CO_2/95% relative humidity) for 48 h to allow the probiotic proteolysis. Incubation at 37 °C resulted in a pH decrease in the samples from 4.25 ± 0.03 to 3.36 ± 0.01 and from 3.87 ± 0.05 to 3.15 ± 0.01, for MB and SB yogurt, respectively.

2.4. In vitro digestion

To simulate gastrointestinal digestion, the method described by Glahn et al. (1998) was applied. Porcine pepsin (P-7000) (800–2500 units/mg protein), pancreatin (P1750) (activity, $4 \times$ USP specifications) and bile extract (B8631) (glycine and taurine conjugates of hyodeoxycholic and other bile salts) were demineralised with Chelex-100 (Bio-Rad Laboratories, Hercules, CA, USA) before use.

After gastric digestion (pH 2), the intestinal digestion (pH 6.9–7) was carried out in the upper chamber of a two-chamber system in 6-well plates (37 °C/5% CO_2/95% air relative humidity). The contents of the upper chamber are separated from the media in the bottom chamber by a semipermeable membrane with a molecular weight cut-off of 15,000 Da, thereby allowing lower molecular weight soluble components to diffuse into the bottom chamber. In the bottom chamber, 1 mL of the isotonic solution [140 mM NaCl, 4 mM KCl] for dialysability assays (without the presence of cells) or minimum essential medium (MEM) for cell uptake experiments was placed, respectively. Control solutions containing digestive enzymes but no sample were used in parallel. After the gastrointestinal incubation period, total Fe content in the bottom chamber

of plates was measured to estimate the dialysable Fe fractions.

2.5. Cell Culture and ferritin analysis in cell monolayer

Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD, USA) at passage 17, and used in experiments at passage 28–32.

For ferritin formation assays, Caco-2 cells were seeded at 50,000 cells cm^{-2} in 6-well collagen treated culture plates (Costar), and were grown with Dulbecco's modified eagle medium (DMEM, Gibco). The experiments were conducted at 14 days post-seeding. Prior to the uptake assay, cultures were washed with tempered (37 °C) MEM at pH 7, and 1 mL of MEM was placed in each well. After intestinal digestion the inserts were removed and an additional 1 mL of MEM was added to each well. Then cell cultures were incubated for additional 22 h.

An immunoradiometric assay was used to measure Caco-2 cell ferritin content (FER-IRON II Serum Ferritin Assay, RAMCO Laboratories, Houston, TX, USA) (Glahn et al., 1998). The concentrations of ferritin were standardized by determination of total protein contents. Total protein content was measured using a Bio-Rad DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Control cells, exposed to in vitro digestion control solutions containing digestive enzymes but no sample, were used throughout. Afterwards, associated Fe content in the harvested cells was quantified by ICP-ES.

2.6. Mitochondrial dehydrogenase activities

Cell viability was evaluated by monitoring MTT (3-[4,5-dimethylthiazol-2-yl]-2,3-diphenyl tetrazolium bromide) conversion on exposed cultures after an incubation period (Laparra, Vélez, Montoro, Barberá, & Farré, 2005). This colorimetric method is based on the reduction of the tetrazolium ring of MTT by mitochondrial dehydrogenases coupled to the phosphorylation process (Ekmekcioglu, Strauss-Blasche, Leibetseder, & Marktl, 1999), yielding a purple formazan product which can be measured spectrophotometrically; the amount of formazan produced is proportional to the number of viable cells. The conversion to insoluble formazan was measured at 570 nm with background subtraction at 690 nm. Control cells, exposed to MEM only, were used through every assay.

2.7. Statistical analysis

A one-factor analysis of variance (ANOVA) and the Tukey test (Box, Hunter, & Hunter, 1978) were applied to determine statistical differences in the mineral contents, dialysability percentages and ferritin formation. All experiments were conducted in triplicate in two different days ($n = 5$). A significance level of $p < 0.05$ was adopted for all comparisons. Statgraphics Plus version 5.1 (Rockville, Md, USA) was used for the statistical analysis.

3. Results and discussion

Inulin used in this study was a mixture of short- and long-chain (degree of polymerization, DP = 3–30) oligo-fructose polymers (Orafti, Belgium). The analysed yogurts showed significant ($p < 0.05$) differences in their Fe contents being 4.8-fold higher in the SB yogurt, 12.6 ± 2.9 vs 60.5 ± 6.9 µg/g sample (dry weight) for MB and SB yogurts, respectively. Neither sample was fortified with Fe.

After the uptake incubation period (24 h), the MTT conversion percentages obtained ranged between 110% and 140% with respect to the controls. These data provide evidence that energetic cell metabolism was not impaired and cell viabilities were adequate during all of the experiments.

3.1. Effects on Fe dialysability

The dialysable Fe fractions obtained in in vitro digestion of yogurts (with and without supplemental 4% inulin), and the dialysability percentages are shown in Table 1. The amount of Fe measured in aliquots that were loaded in the upper chamber was consistent with the expected content of the digests. In non-incubated samples, after addition of inulin the dialysable Fe fraction in the MB yogurt was decreased, but dialysable Fe in the SB yogurt was increased. For MB, a reduction ($p < 0.05$) of 56.9% was observed, meanwhile, for SB a marked increase ($p < 0.05$) was noted (5-fold). Incubation of samples at 37 °C without the addition of inulin, produced increasing dialysable Fe in both yogurts. Interestingly, dialysable Fe was higher in incubated SB samples containing added inulin but not in MB yogurt. The most likely explanation might be that proteolysis caused by probiotic bacteria present in both yogurts during incubation period yielded low-molecular-weight peptides that could bind mineral ions, keeping them in solution. However, SB yogurt fermentation in the presence of inulin had no effect on dialysable Fe relative to non added sample. As shown, dialysable Fe content in SB yogurt with added inulin was similar before and after fermentation (I+/F− vs I−/F+).

When the results are expressed as a percentage of the initial amount of Fe loaded in the upper chamber of in vitro system, i.e, as dialysability (see calculations in Table 1), without the addition of inulin the MB yogurt showed a value of 6.3-fold higher ($p < 0.05$) than SB yogurt. The higher dialysability percentages obtained for MB yogurt (I−/F−) might be due to a more efficient capability of the proteolytic enzymes in the in vitro digestion for producing low-molecular-weight peptides able to keep Fe in solution. In this sense, the production of low-molecular-weight bioactive casein phosphopeptides (CPPs) which could act as mineral carriers (Bouhallab

Table 1
Dialysable Fe and dialysability percentages in milk- and soy-based yogurts

Sample	Treatment[A]	Fe in the upper chamber (µg)	Dialysable Fe (µg)	Dialysability[A] (%)
Milk-based	I(−)/F(−)	0.266 ± 0.029^a	0.072 ± 0.001^b	27.2 ± 3.0^b
	I(+)/F(−)	0.254 ± 0.012^a	0.031 ± 0.004^a	12.0 ± 1.2^a
	I(−)/F(+)	0.266 ± 0.026^a	0.105 ± 0.020^c	$39.6 \pm 9.2^{b,c}$
	I(+)/F(+)	0.247 ± 0.016^a	0.131 ± 0.008^c	54.0 ± 8.7^c
Soy-based	I(−)/F(−)	1.249 ± 0.039^a	0.053 ± 0.005^a	4.3 ± 0.3^a
	I(+)/F(−)	1.213 ± 0.064^a	0.276 ± 0.042^c	22.7 ± 2.2^c
	I(−)/F(+)	1.213 ± 0.058^a	0.130 ± 0.015^b	10.7 ± 0.9^b
	I(+)/F(+)	1.198 ± 0.061^a	0.330 ± 0.069^c	27.7 ± 6.7^c

Results are expressed as mean value ± standard deviation ($n = 4$). Within milk or soy-based samples, values with no letter in common indicate statistically significant ($p < 0.05$) differences between dialysable Fe and dialysability (%). Treatments: I(−): no added inulin; I(+): 4% (w/w) added inulin; F(−): no incubation; F(+): incubated at 37 °C for 48 h.
[A] Calculated as: (Dialysable Fe/Fe in the upper chamber) × 100.

et al., 2002), cannot be ruled out. However, the Fe dialysability was impaired by the presence of inulin only in MB yogurt. It may be hypothesized that there is an interaction of inulin with CPPs and impairing their dialysability. This observation would be in accordance with a previous report where the authors evidenced that inulin (supplemental 3%) impaired Fe dialysability from a milk-based infant formula (Bosscher et al., 2003). Taking into consideration that our in vitro model fits a semipermeable membrane to create a bicameral system, the dialysability percentages calculated for MB yogurt (I+/F+) may indicate the equilibrium in the dialysis process. This result supports our suggestion of higher proteolysis occurring in MB yogurt. On the other hand, in both non-incubated and incubated SB yogurts, samples with added inulin exhibited an increased Fe dialysability (Table 1). The latter observation suggests higher soluble Fe contents which were able to cross the dialysis membrane used (15,000 Da). These data are in good agreement with a previous study of our group (Yasuda et al., 2006), where the authors reported that corn-soy diets supplemented with inulin had higher soluble Fe concentrations in the proximal, mid and distal colon of piglets compared with controls. Furthermore, this fact was accompanied by an enhanced utilization of intrinsic Fe (Yasuda et al., 2006).

Presently, there are few studies on Fe availability in different type of yogurts or the possibility to fortify these with several Fe sources. It is also interesting to mention that the Fe dialysability estimated in this study for the MB yogurt was higher than the 4.37% previously reported for a FeSO$_4$ fortified MB yogurt, but without live probiotic cultures (Drago & Valencia, 2002). Unfortunately, data related to the effect of soy-milk digestion products on Fe availability have been obtained only with infant formula (Bosscher et al., 2001; Nogueira, Colli, & Amancio, 2005). The latter studies reported higher Fe dialysability percentages in soy-based than in casein-based infant formula, 1.5% vs 0.5% (Bosscher et al., 2001) and 5.3% vs 3.6% (Nogueira et al., 2005). Differences in the in vitro methods employed could account for much of large different percentages reported between these studies.

Yogurt and fermented milk are frequently promoted as carriers of probiotic bacteria, and the proteolysis of milk-caseins by probiotic bacteria has been extensively studied (Abu-Tarboush, 1996; El-Zahar, Chobert, Dalgalarrondo, Sitohy, & Haertlé, 2004; Fedele, Seraglia, Battistotti, Pinelli, & Traldi, 1999). However, previous studies have reported that soy milk supports the growth of lactic acid bacteria (L. casei, L. salivarus) (Brink, Todorov, Martin, Senekal, & Dicks, 2006) and several Bifidobacterium sp. (B. infantis, B. longum, B. bifidum and B. adoslescentis) (Kamaly, 1997), but little work has been carried out on the proteolysis derived products in soy-milk. Otherwise, it is well known that some casein-derived proteolysis peptides, produced during gastrointestinal digestion, improve Fe availability (Bouhallab et al., 2002), but there is lack of data on the effect of soy-derived proteolysis products produced in the gut on Fe availability.

3.2. Ferritin formation and cell associated Fe content

Once Fe is internalised in the cell, ferritin constitutes the main intracellular Fe pool (Arredondo, Orellana, Gárate, & Núñez, 1997). Ferritin concentrations in cells exposed to digests of yogurts, are shown in Fig. 1. Lower ferritin contents were detected in cell cultures exposed to the digests of MB yogurt with added inulin. These data reflect differences in dialysable Fe mentioned above. As stated before, the possible interaction of inulin with CPPs might explain the decreased Fe availability to the cultured cells in MB yogurt. Comparing the ferritin concentrations in both yogurts, with and without inulin, cells exposed to MB yogurt exhibit higher ($p < 0.05$) ferritin formation than those incubated with digests of SB yogurt. For the latter, no statistically ($p > 0.05$) significant differences with or without inulin were detected. It is important to mention that ferritin formation in cultures exposed to non-fermented SB yogurt with added inulin, did not correspond to the higher dialysable Fe content suggesting that soluble Fe-complexes are not available for cell uptake in this sample. The absence of significant ($p > 0.05$) differences in ferritin concentrations supports this hypothesis.
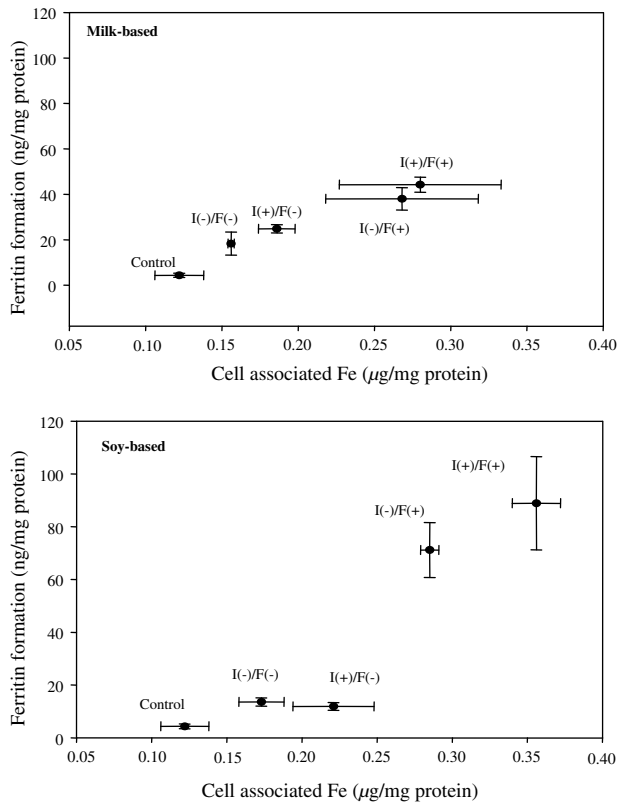
Fig. 1. Plot of cell associated Fe vs. cell ferritin measured 24 h after the start of the intestinal digestion period. Values are expressed as mean ± standard deviation ($n = 5$). I(−): no added inulin; I(+): 4% (w/w) added inulin; F(−): no incubation; F(+): incubated at 37 °C for 48 h.

Following incubation (37 °C/48 h), for both MB and SB yogurts with and without inulin, ferritin formation was higher ($p < 0.05$) with respect to non-fermented samples. The enhancing effect of incubation on Fe availability observed is in accordance with a previous study (Bergqvist, Andlid, & Sandberg, 2006), where the authors reported an increased Fe solubility and uptake by Caco-2 cells from lactic acid fermentation of carrot juice. The authors attributed this effect to pH-mediated influence on soluble Fe and the high Fe^{2+} quantified in the soluble fraction. However, it should be pointed out that casein was ineffective in reducing Fe^{3+} in vitro (Kapsokefalou & Miller, 1991). In this sense there are no studies on the effect of soy components on the oxidation state of soluble Fe.

Ferritin expression is translationally regulated by intracellular iron concentration (Pantopoulos, 2004; Rouault, 2006), and formation by intestinal cells occurs in response to Fe that has been taken up. Therefore, it was of interest to ascertain the variation in intracellular levels of ferritin as a function of the cell associated Fe contents (Fig. 1). For non fermented samples, cell Fe increased with increases in dialysable iron in cells exposed to digests of MB yogurt. However, in cells exposed to SB yogurt with supplemental inulin, the increase in dialysable Fe was not reflected in higher cell Fe content. These data suggest that dialysable Fe was not equally available from both types of yogurt. After incubation the cell Fe content from any SB sample, with or

without added inulin, was 2.4- and 3.1-fold higher, respectively, compared to non-fermented samples. It is interesting to mention that addition of inulin caused a 2-fold increase in dialysable Fe and cell Fe content, without differences in ferritin concentration quantified in these cultures.

In a previous study (Glahn et al., 1995), we showed that insoluble Fe forms could be nonspecifically bound to surface proteins causing overestimations in Fe uptake values. The authors emphasized the need to consider the solubility of Fe, because soluble forms of Fe were less likely to bind to the surface of Caco-2 cells. In the present study a dialysis membrane was used to separate insoluble Fe in the digests from reaching the lower chamber. Thus, it could be expected that surface-bound Fe would represent a low proportion of the cell associated Fe. However, as shown, a high variability (33.9%) in cell associated Fe was observed in cultures exposed to MB digests. The non-incubated MB samples exhibit a considerably lower variability of 4.3% and 19.3%, without and with inulin in the media, respectively. These differences could be attributed to the existing differences in proteolysis products caused by enzymes produced by the bacteria. In this way, Miquel et al. (2005) have reported the formation of specific monophosphorylated peptides from probiotic-containing infant formula after applying a similar in vitro digestion procedure to that used in this study. The authors indicated the possible formation of these specific casein-derived peptides by bifidobacteria sp, based on the absence of reports supporting the possibility to be produced as a result of pancreatic digestion. Otherwise, the low variability (mean value of 5.2%, without and with inulin) in the cell associated content from SB might suggest a food matrix effect in the Fe-bound to cell surface. The reported effects when studying Fe availability in foods, should be considered to avoid overestimation in Fe uptake if only analytical methods to quantify cell associated Fe are used. It is also important to point out that, although solubility is a prerequisite to be absorbed not all soluble forms of Fe are available to Caco-2 cells as concluded from ferritin formation values quantified.

Also of interest is the lack of a significant ($p > 0.05$) difference in ferritin formation between the digests from non-incubated MB, and both non-incubated and incubated SB. It must be pointed out that a two-fold increase in cell associated Fe contents, did not translate to an increase in ferritin formation by Caco-2 cells. This observation would be in accordance with our previous study (Glahn et al., 1998), where Caco-2 cultures exposed to digests containing $FeSO_4$ combined with ascorbic acid (50 and 100 μM) exhibited a similar ferritin formation within a 1.5-fold higher cell associated Fe. In the latter study, plotting cell Fe content versus cell ferritin resulted in a nonlinear correlation. However, the same plot displayed a linear correlation when $FeSO_4$ was combined with citric acid (Glahn et al., 1998). In addition, Arredondo et al. (1997) also reported a nonlinear ferritin formation by Caco-2 cells with a steeper increment at intracellular iron concentrations of 24–114 μM. The authors suggested that this observation might be explained

by the high binding ability of each ferritin molecule for up to 4500 Fe atoms (Arredondo et al., 1997).

It is important to bear in mind that iron regulatory proteins (IRP) register cytosolic iron concentrations and post-transcriptionally regulate the expression of genes involved in cellular Fe storage and transport (Rouault, 2006). Johnson et al. (2005) indicated that high levels of Fe present in the apical surface of intestinal epithelia caused a relocalization of DMT1 transporter, and limiting the cell Fe internalization. However, in the present study, similar ($p > 0.05$) Fe concentrations in the digests only caused significant ($p > 0.05$) differences on ferritin formation after incubation of SB. This observation suggests an enhancing effect on Fe uptake by probiotic bacteria, which is in good agreement with a previous study (Bergqvist et al., 2006).

In summary, the addition of 4% inulin did not alter the uptake of nonhaem-iron by Caco-2 cells. While *in vitro* results are not completely extrapolable to the in vivo situation, the data presented appear to suggest that inulin does not have a direct effect on Fe bioavailability in the small intestine. These results are in accordance with a study on nonanemic humans reporting that indigestible oligosaccharides do not interfere with nonhaem-iron absorption (Van den Heuvel et al., 1998). However, they appear to conflict with reports that supplemental inulin enhances iron absorption in piglets (Yasuda et al., 2006). This may indicate that the mechanism(s) whereby inulin enhances iron absorption in anemic piglets involves the large intestine. Our *in vitro* model mimics events in the small intestine but does not simulate the large intestine. In the present study, those samples incubated for 48 h to simulate large the fermentation processes that can occur in large intestine produced higher cell ferritin values suggesting that probiotic activity can render more available Fe. This suggestion would be in accordance with those data previously reported by Yasuda et al. (2006). Therefore, intestinal microflora and probiotic supplementation, are aspects that would be taken into account in order to more reliable estimation of Fe uptake associated to consumption of foods. However, additional studies are needed to confirm the probiotic effect and the involvement of large intestine on Fe absorption. Improved understanding of the effects and mechanisms of prebiotics such as inulin upon Fe availability are needed to design adequate dietary strategies.

Acknowledgements

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