

## Lactic acid fermentation stimulated iron absorption by Caco-2 cells is associated with increased soluble iron content in carrot juice

Sharon W. Bergqvist\*, Thomas Andlid and Ann-Sofie Sandberg

Department of Chemical and Biological Engineering/Food Science, Chalmers University of Technology, Box 5401, 402 29 Gothenburg, Sweden

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An *in vitro* digestion/Caco-2 cell model was applied to explore the impact of lactic acid (LA) fermentation by *Lactobacillus pentosus* FSC1 and *Leuconostoc mesenteroides* FSC2 on the Fe bioavailability of carrot juice. The redox state of Fe in fermented carrot juice was also assessed as a crucial factor for absorption. LA fermentation was shown to improve mineral solubility to different extents at simulated physiological conditions: Mn (2-fold); Fe (1.5–1.7-fold); Zn (1.2-fold); Cu (1-fold). Soluble Fe<sup>2+</sup> was increased about 16-fold by LA fermentation, and about one third of the Fe<sup>2+</sup> remained soluble after *in vitro* digestion (about 4–5-fold higher than in fresh juice). Data on cell-line studies showed a 4-fold increase in the efficiency of Fe uptake, but not in transepithelial transfer by Caco2 cells, as a result of fermentation. The increases in Fe<sup>2+</sup> level and the efficiency of cellular Fe uptake were strain-dependent. To sum up the effect on both Fe solubility and cellular uptake efficiency, the amount of cellularly absorbed Fe from *Ln. mesenteroides* FSC2-fermented juice was about 20% higher than that from *L. pentosus* FSC1-fermented juice (22.7 v. 19.2 µg/l juice per mg protein). To conclude, LA fermentation enhanced Fe absorption by Caco-2 cells from carrot juice because of increases in not only Fe solubility after digestion, but also the efficiency of cellular Fe uptake. The fermentation-improved efficiency of Fe uptake was possibly due to the increased level of soluble Fe<sup>2+</sup> rather than a being a strain-specific event.

### Iron absorption: Iron redox state: Caco-2 cells: Lactic acid fermentation

Fe is essential for mammals. Its homeostasis in human subjects is strictly regulated by its absorption at the intestinal mucosa as active excretion is lacking. The daily Fe loss (about 1–2 mg) must thus be compensated through dietary intake. Inadequate absorption leads to Fe deficiency, consequently resulting in anaemia and a delay of growth and intellectual development (Umbreit, 2005). Half of the world's population is currently suffering from Fe deficiency as a result of low Fe bioavailability in many types of staple food. Strategies to improve dietary Fe bioavailability therefore become important.

Dietary Fe, existing as haem and non-haem Fe, needs to be in a soluble state before absorption from the lumen of the gut. Non-haem Fe, contributing about 90% of the total Fe source, mostly presents in complexes with other food compounds, occurring in cereals, vegetables, legumes and fruits. It has two distinct redox states: ferrous (Fe<sup>2+</sup>); ferric (Fe<sup>3+</sup>) Fe. Fe<sup>2+</sup> is the major form absorbed by the enterocytes (Wollenberg & Rummel, 1987; Han *et al.* 1995). It tends, however, to be oxidised to Fe<sup>3+</sup> in an aerobic environment, unless protected by reducing agents. Fe<sup>3+</sup>, on the other hand, is the most abundant form of non-haem Fe. It is readily precipitated as Fe(OH)<sub>3</sub> if not chelated in soluble complexes at the absorption site.

Some known dietary factors, such as phytate (Han *et al.* 1994) and polyphenols (Gillooly *et al.* 1983), inhibit Fe

absorption by forming insoluble complexes with Fe under physiological pH conditions, whereas ascorbic acid (Hallberg *et al.* 1989) may increase the bioavailability of Fe by both chelating Fe and reducing Fe<sup>3+</sup> to Fe<sup>2+</sup>. In addition, gastrointestinal digestion may also affect the availability of Fe by influencing the release of Fe from the food matrix through the action of digestive enzymes, and Fe solubility through its physiological changes in pH in the stomach and intestinal phases (Beard *et al.* 1996). Moreover, some dietary factors may influence Fe absorption by interfering with mucosal Fe-uptake pathways. One example is that some other divalent minerals, for example Mn, Zn and Cu, may limit luminal Fe absorption by competing with Fe<sup>2+</sup> for the uptake protein divalent metal transporter-1 (Gunshin *et al.* 1997; Yeung *et al.* 2005). Unfortunately, this area remains largely unknown as the metabolism of intestinal Fe absorption is not yet totally understood.

Carrots are one of the major vegetables consumed during all seasons. They constitute about 10% of the vegetables consumed in Sweden (National Food Administration, 2002). Besides being one of the richest vegetable sources of viscous soluble dietary fibre, they also contain antioxidants, in particular, carotenoids, as well as vitamins C and E, phenolic compounds and phytate (Alasalvar *et al.* 2001). Their soluble dietary fibre is known to lower plasma cholesterol (Mazur

*et al.* 1990; Fernandez *et al.* 1995) and postprandial glucose levels and insulin response (Torsdottir *et al.* 1991, 1992). Similarly, the antioxidants contribute to antioxidant protection (Miller *et al.* 1996; Rice-Evans *et al.* 1996) and furthermore influence Fe bioavailability (Brune *et al.* 1991; Hurrell *et al.* 1999; Garcia-Casal *et al.* 2000).  $\beta$ -Carotene has been suggested to increase Fe absorption and to overcome the inhibitory effects of polyphenols and phytate by forming soluble complexes with Fe (Garcia-Casal *et al.* 2000). Apart from Fe, carrots are a source of other essential dietary minerals, for example Ca, Mg, Zn and Se.

Fresh carrot juice is a popular commercially available beverage in Sweden but suffers from the disadvantage of having a short shelf-life. There are several possible advantages to applying lactic acid (LA) fermentation to carrot juice. First, it is well proven as a safe biotechnology for food (Stiles, 1996). Second, the reduction in pH, enzymatic degradation of Fe-containing macromolecules and chelation by organic acids produced by the LA bacteria (LAB) may improve Fe availability (Teucher *et al.* 2004). Third, LAB have the unusual property of no or a low requirement for Fe (Archibald, 1986; Pandey *et al.* 1994) and will therefore not accumulate Fe or compete for the available Fe with human enterocytes; if a micro-organism is consumed as a live cell it is not necessarily lysed, and nutrients in its cytoplasm may never be absorbed in the intestine. Fourth, inhibitors of Fe absorption such as phytate and tannins have been shown to be degraded during LA fermentation (Svanberg *et al.* 1993; Bergqvist *et al.* 2005b). Finally, shelf-life is increasing as a result of natural biopreservation, and, at least in theory, flavour may improve. However, a decreased uptake of  $\text{Fe}^{3+}$  in the presence of LA (Bergqvist *et al.* 2005a) and citric acid (Narasima Rao & Subba Rao, 1992; Glahn *et al.* 1998) has been reported. The reason may be that Fe-chelating molecules with a high affinity, such as citrate, may compete with luminal uptake proteins for attracting the Fe.

In our previous work, a strain-dependent increase in soluble Fe in carrot juice fermented by *Lactobacillus pentosus* FSC1 and *Leuconostoc mesenteroides* FSC2 was obtained (Bergqvist *et al.* 2005b). The strains had previously been selected for yielding a high increase in Fe solubility. However, whether this effect would remain at the site of intestinal absorption after gastrointestinal digestion is not yet clear. Furthermore, the valence states of Fe in the fermented juice were of interest as  $\text{Fe}^{2+}$  is the more favoured form for absorption compared with  $\text{Fe}^{3+}$ . In the present study, a combined model of *in vitro* digestion and uptake by Caco-2 cells was applied to study Fe bioavailability in LA-fermented carrot juice. The impact of LA fermentation on the level of  $\text{Fe}^{2+}$  in carrot juice during gastrointestinal digestion was also investigated.

## Materials and methods

### Reagents

All reagents were from obtained GTF (Göteborg, Sweden) unless otherwise noted. The radioisotope ( $^{55}\text{FeCl}_3$ ) was purchased from Perkin-Elmer (Billerica, MA, USA).

### Caco-2 cell culture

Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD, USA) at passage 17 and used for experiments within the following fifteen passages. Stock cultures were maintained in Dulbecco's modified alpha essential medium supplemented with 16% (v/v) fetal calf serum, 100 U/l penicillin G and 100 mg/l streptomycin at 37°C in a humidified atmosphere of 95% air and 5%  $\text{CO}_2$ . Fetal calf serum (16%) was used in the stock media based on the finding (Gangloff *et al.* 1996) that there was 4.37  $\mu\text{mol/l}$  Fe in this medium with 10% fetal calf serum. The growth medium was changed every second or third day. Cells were split at approximately 80% confluence using 0.5 g/l trypsin with 0.2 g/l EDTA in PBS.

Prior to the experiments, 100 000 cells in a volume of 0.5 ml supplemented Dulbecco's modified  $\alpha$  essential medium were seeded onto 0.4  $\mu\text{m}$  microporous membrane inserts (1  $\text{cm}^2$  Transwell inserts; Corning, Acton, MA, USA). The basolateral chamber contained 1.5 ml supplemented medium. The medium on both sides of the filter insert was changed every 2–3 d. Transepithelial electrical resistance was monitored to assess the formation of a tight cell monolayer, and the formation of fully differentiated cell monolayer was typically established by day 10 (transepithelial electrical resistance increasing from about 100 to 250  $\Omega/\text{cm}^2$ ; data not shown). All Fe uptake and transfer experiments were performed at 15–18 d after seeding. Fresh supplemented Dulbecco's modified  $\alpha$  essential medium was provided to the cells 12 d prior to the uptake and transfer assays.

### Carrot juice preparation and fermentation

The carrots used in the present study were purchased from the local market. The same batch was used in all experiments. Carrot juice was prepared and fermented as previously described (Bergqvist *et al.* 2005b). Briefly, the carrots were washed, peeled and homogenised by Multipress automatic MP 80 (Braun, Kronberg, Germany) and yielded into solid and liquid phases. The solid material of the homogenised carrots was added back to the liquid phase and then incubated with Pectinex Ultra SP-L (containing pectolytic enzyme with a standard activity of 26 000 PG/ml at pH 3.5) and Celluclast 1.5 litre (containing cellulase with an activity of 1800 NCU/ml) (Novo Nordisk A/S) at a concentration of 4 ml/l juice for 1 h at 40°C. The juice was then stored at  $-20^\circ\text{C}$  until use for fermentation. The levels of total minerals in the carrot juice were quantified as previously described (Bergqvist *et al.* 2005b; 2.03, 0.88, 0.32 and 0.52  $\mu\text{g/g}$  for Fe, Zn, Mn and Cu, respectively). Similar values were obtained in the fermented juice.

The strain selection, *L. pentosus* FSC1 and *Ln. mesenteroides*, was based on a previous screening for a high ability to solubilise Fe and for representing homofermentative and heterofermentative LAB, respectively. Homofermentative LAB (*L. pentosus*) have LA as the major end product, whereas heterofermentative LAB (*L. mesenteroides*) produce a mixture of LA, acetic acid and ethanol as fermentation end products.

*L. pentosus* FSC1 and *Ln. mesenteroides* FSC2 were precultured in de man, Rogosa, Sharpe broth (Fluka, Buchs, Switzerland) for 15 h in a rotary shaker (Labline Instruments

Inc., Melrose Park, IL, USA) at 30 °C and 200 rpm. Cultures were harvested and washed twice with sterile 0.9% NaCl and inoculated in 100 ml carrot juice in an E-flask to obtain an initial cell concentration in the juice equivalent to an optical density of 0.2 (about  $10^9$  colony-forming units/ml for *L. pentosus* FSC1 and  $10^8$  colony-forming units/ml for *Ln. mesenteroides* FSC2). Prior to fermentation, the carrot juice was thawed at room temperature and then adjusted to pH 6 by 5 or 1 mol/l NaOH. Fermentations were performed on a rotary shaker (TECHTUM LAB, Riga, Latvia) for 24 h at 200 rpm and 30 °C. Both fresh (pH 6) and fermented juice were stored at -20 °C until use for *in vitro* digestion. After fermentation, the cell concentration of both strains had increased to about ten times the inoculated start level as determined by measuring the number of colony-forming units, and the pH had dropped to 4.0 and 3.9 for *L. pentosus* FSC1 and *Ln. mesenteroides* FSC2, respectively. The total acidity in the fermented juice was also controlled by titration with 0.1 mol/l NaOH. The titration data strongly pointed to successful homofermentation and heterofermentation by the two LAB (data not shown).

#### *In vitro* digestion and mineral availability

The method used was that described by Au and Reddy (2000), with slight modification. Briefly, each carrot juice (10 ml) was adjusted to pH 2 with 5 mol/l HCl, and 0.3 ml freshly made pepsin (P7000; Sigma, St Louis, MO, USA) solution (0.16 g (1130 U/mg protein) per 0.1 mol/l HCl) was added. The mix was incubated on a rotary shaker at 90 rpm at 37 °C in a humidified atmosphere of 95% air and 5% CO<sub>2</sub> for 1 h. After simulated gastric digestion, the pH of the samples was brought to 6 by the dropwise addition of 1 mol/l NaHCO<sub>3</sub>. A volume of 1.7 ml pancreatin–bile mixture (0.12 g bile extract and 0.02 g pancreatin (4 × USP activity, preincubated under the same condition as for gastric digestion) in 5 ml 0.1 mol/l NaHCO<sub>3</sub>) was mixed with each sample and incubated for 0.5 h at 90 rpm at 37 °C in a humidified atmosphere of 95% air and 5% CO<sub>2</sub>. The digests were placed on ice to stop pancreatic activity. Volumes of all samples were brought to 15 ml and then centrifuged at 5000 g for 30 min at 4 °C. The soluble minerals were quantified according to Fredrikson *et al.* (2002), by centrifuging a mixture of 0.8 ml supernatant with 1 ml 20 g/l ascorbic acid and 0.1 ml 0.5 mol/l HCl at 9300 g for 10 min and analysed by high-performance ion chromatography. The availability of Cu, Zn, Mn and Fe in the juice was defined as the soluble fraction of each mineral after *in vitro* digestion.

#### *Fe* uptake and transfer by Caco-2 cells

A mix of 2 ml digested supernatant and 2 ml Hanks' Balanced Salt Solution was used for Fe uptake and transepithelial transfer by Caco-2 cells to avoid the possible toxic effect of a high concentration of the organic acids in the fermented juice. Radio-labelled <sup>55</sup>FeCl in 0.01 mol/l HCl was then added to each of the 4 ml samples (containing 2 ml supernatant from the digest) to obtain 0.4 MBq per sample (contributing about 5% of the total Fe). Volumes of the extrinsically labelled sample solutions (0.01 ml) were set aside for measurement of initial radioactivity.

Prior to the assay, the Caco-2 cells were washed once with Hanks' Balanced Salt Solution. Samples with <sup>55</sup>Fe were allowed to equilibrate for 15 min before being applied to the Caco-2 cells. Aliquots of 0.5 ml from each sample were placed on the apical side of the Caco-2 cells, whereas the basolateral chamber contained 1.5 ml Hanks' Balanced Salt Solution. Cells were incubated for 1 h at 37 °C in a humidified atmosphere of 95% air and 5% CO<sub>2</sub>, and 1 ml solution was removed immediately to measure the radioactivity. The cells were washed three times with ice-cold washing buffer (150 mmol/l NaCl, 10 mmol/l HEPES, 1 mmol/l EDTA; pH 7) and homogenised in PBS with 1 mmol/l EDTA and 0.2% Triton X-100 added. Protein determinations were accomplished by the Bradford assay (Bio-Rad, Sundbyberg, Sweden). <sup>55</sup>Fe transferred to the basolateral chamber and associated with the Caco-2 cell lysates was measured by liquid scintillation counting, and its percentage in terms of the total <sup>55</sup>Fe applied to each insert was expressed as the efficiency. The integrity of the cell monolayers was monitored before and after the assays by measuring transepithelial electrical resistance, and leaky cell monolayers (indicated by a decline in transepithelial electrical resistance value) were discarded.

#### *Quantification of soluble Fe<sup>2+</sup> in carrot juice*

The level of Fe<sup>2+</sup> in the carrot juice before digestion, after gastric digestion (pH 2) and after intestinal digestion (pH 6) was assessed by spectrophotometrical measurement of its complex with bathophenathrolinesulfonate at 535 nm according to Cowart *et al.* (1993) and Avron (1963). Briefly, after 10 min centrifugation at 9300 g, each supernatant (0.2 ml) was diluted to a total volume of 0.95 ml and mixed with 0.05 ml 3.34 g/l bathophenathrolinesulfonate. To obtain a standard curve, 0.5–4 μmol/l FeSO<sub>4</sub> was mixed with bathophenathrolinesulfonate. The formation of Fe<sup>2+</sup>–bathophenathrolinesulfonate was measured spectrophotometrically at 535 nm after 10 min. The level of Fe<sup>2+</sup> was quantified based on the standard curve.

#### *Statistics*

Two independent fermentations by each of the strains *L. pentosus* FSC1 and *Ln. mesenteroides* FSC2 were performed in triplicate within the experiments. Each of the triplicates was digested on three experimental occasions. Fe uptake and transfer from each digest were calculated from average values from three inserts. Data on mineral availability and Fe uptake and transepithelial transfer were thus presented as means and standard deviations of eighteen measurements. Measurements were analysed by ANOVA using the Tukey *post-hoc* test using SYSTAT software (SYSTAT Inc., Evanston, IL). Differences were considered significant if  $P \leq 0.05$ .

## **Results**

### *Lactic acid fermentation improves Fe, Mn and Zn solubility after in vitro digestion*

After the simulated gastric and intestinal digestion, the availability of minerals, including Fe, Mn, Zn and Cu in the juices,

**Table 1.** Impact of lactic acid fermentation on soluble mineral content (mg/l) in carrot juice after *in vitro* gastrointestinal digestion

(Means values and standard deviations for eighteen determinations)

	Cu		Zn		Mn		Fe	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Control	0.40	0.09	0.90	0.09	0.22	0.06	0.98	0.08
<i>Lactobacillus pentosus</i> FSC1	0.40	0.09	1.13	0.08*	0.48	0.07*	1.49	0.16*
<i>Leuconostoc mesenteroides</i> FSC2	0.36	0.09	1.13	0.13*	0.54	0.09*	1.68	0.17*†

Fresh and fermented carrot juice was digested by pepsin digestion at pH 2 for 60 min. This was followed by pancreatin–bile digestion at pH 6 for 30 min as described on page 706. The availability of minerals was defined as the soluble fraction of minerals after *in vitro* digestion.

\* Mean values of each soluble mineral in the fermented juice were significantly different from those in the control juice ( $P \leq 0.05$ ).

† Mean values of each soluble mineral in the fermented juice were significantly different between the two strains ( $P \leq 0.05$ ).

was evaluated by high-performance ion chromatography. As shown in Table 1, fermentation by both *L. pentosus* FSC1 and *Ln. mesenteroides* FSC2 improved the availability of Zn, Mn and Fe but not Cu. The enhancement for both fermented juices could be ranked in the order Mn > Fe > Zn for both strains in their respective fermented juice. Noticeably, changes in the level of available minerals in the fermented juice from the two LAB strains was comparable except for Fe. There was more available Fe in carrot juice fermented by *Ln. mesenteroides* FSC2 than by *L. pentosus* FSC1 (70% v. 50% improvement, respectively;  $P = 0.005$ ).

#### Lactic acid fermentation increases level of Fe<sup>2+</sup> in carrot juice

As the valence state of Fe is a crucial factor in its bioavailability, the influence of LA fermentation on the valence state of Fe in carrot juice was evaluated. Furthermore, the impact of gastrointestinal digestion on the level of soluble Fe<sup>2+</sup> was also studied. As shown in Table 2, fermentation by both *L. pentosus* FSC1 and *Ln. mesenteroides* FSC2 significantly ( $P = 0.001$ ) increased the level of soluble Fe<sup>2+</sup> in carrot juice (16-fold and 18-fold, respectively). Although there was a similar increase in soluble Fe<sup>2+</sup> in the fresh juice as in the two fermented juices during gastric digestion, this increase did not remain after intestinal digestion. The content of soluble Fe<sup>2+</sup> in fresh juice was not significantly influenced by the *in vitro* digestion ( $P = 0.86$ ). Interestingly, a significant

decrease in the level of soluble Fe<sup>2+</sup> was noticed in *L. pentosus* FSC1-fermented juice after gastric digestion (0.84 v. 1.07 mg/l,  $P = 0.05$ ), whereas the level of soluble Fe<sup>2+</sup> remained similar in *Ln. mesenteroides* FSC2-fermented juice (1.19 v. 1.21 mg/l). Although the Fe<sup>2+</sup> content of both fermented juices was significantly decreased after intestinal digestion ( $P \leq 0.001$ ), about one third of the Fe<sup>2+</sup> that was reduced during fermentation was retained. The level of available Fe<sup>2+</sup> in *L. pentosus* FSC1-fermented juice and *Ln. mesenteroides* FSC2-fermented juice was still 5-fold and 4-fold higher than that of the fresh juice after *in vitro* digestion (0.45 and 0.38 v. 0.09 mg/l), accounting for about 30% and 23% of the total available Fe, respectively. Nevertheless, the Fe<sup>2+</sup> content of the fermented juice at each digestion phase was not significantly different between the two strains.

#### Lactic acid fermentation increases efficiency of Fe uptake but not transepithelial transfer by Caco-2 cells

The cellular uptake and transepithelial transfer of Fe from digested carrot juice were evaluated as described in Methods. The intracellular and transported radioactive Fe as a percentage of the total radioactivity applied at each insert was calculated and expressed as efficiency. As shown in Fig. 1, the uptake of Fe from fermented juice was about 4-fold more efficient than that from fresh juice, whereas the efficiency of Fe transfer across the cell monolayer from both fermented juices remained similar when compared with fresh juice.

**Table 2.** Impact of lactic acid fermentation on the level of soluble Fe<sup>2+</sup> in carrot juice during gastrointestinal digestion

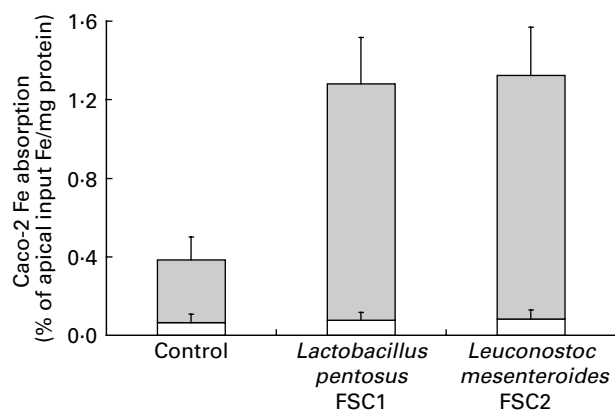
(Means values and standard deviations for twelve determinations)

	Fe <sup>2+</sup> (mg/l)					
	Before digestion		Gastric digestion		Intestinal digestion	
	Mean	SD	Mean	SD	Mean	SD
Control	0.07	0.02	1.03	0.04 <sup>a</sup>	0.09	0.02
<i>Lactobacillus pentosus</i> FSC1	1.07	0.14*	0.84	0.07 <sup>a</sup>	0.45	0.20* <sup>b</sup>
<i>Leuconostoc mesenteroides</i> FSC2	1.21	0.11*	1.19	0.28	0.38	0.13* <sup>a</sup>

Fresh and fermented carrot juice was digested by pepsin digestion at pH 2 for 60 min. This was followed by pancreatin–bile digestion at pH 6 for 30 min as described on page 706.

\* Mean values of soluble Fe<sup>2+</sup> in the fermented juice were significantly different from those in the control juice within the same digestion step ( $P \leq 0.05$ ; comparison within each column).

<sup>a,b</sup> Mean values of soluble Fe<sup>2+</sup> after each digestion step were significantly different from those before digestion within the same juice. Mean values within a row with unlike superscript letters were significantly different between the two digestion steps ( $P \leq 0.05$ ).



**Fig. 1.** Lactic acid fermentation improves efficiency of Fe uptake (▣) but not transepithelial transfer (□) from carrot juice by Caco-2 cells. Both fresh and fermented carrot juice were digested before the addition of a trace amount of  $^{55}\text{FeCl}$ . A 0.5 ml aliquot from each digest was applied to the apical side of a 15–17 d post-seeding Caco-2 cell monolayer. After 1 h incubation, non-specifically bound Fe was removed from the apical membrane.  $^{55}\text{Fe}$  transferred to the basolateral chamber and associated with the Caco-2 cell lysates was measured by liquid scintillation counting. Cell-associated radioactivity was normalised to the protein content measured for each insert, and their percentage of the total  $^{55}\text{Fe}$  applied to each insert was expressed as efficiency. Values are means and standard deviations ( $n$  18). Mean values were significantly different from those of the control group ( $P \leq 0.05$ ).

Noticeably, the efficiency of Fe uptake stimulated by fermentation by the two LAB strains was not significantly different ( $P = 0.795$ ).

When including the improvement in both Fe availability and uptake efficiency, the overall amount of cellular Fe uptake from *L. pentosus* FSC1 and *Ln. mesenteroides* FSC2 fermented juice was 6-fold and 7-fold higher than that from fresh juice (18 and 21 v. 3  $\mu\text{g/l}$  juice/mg protein, respectively). The mean values for Fe transferred across the cells were also 2-fold and 2.5-fold greater in fermented juice than fresh juice (1.2 and 1.5 v. 0.6  $\mu\text{g/l}$  juice/mg protein, respectively), although this increase did not reach statistical difference.

## Discussion

The present study revealed that the fermentation of carrot juice by LA improved the availability of several minerals, such as (in descending order) Mn, Fe and Zn, but not Cu. The uptake of Fe by Caco-2 cells was also stimulated by LA fermentation. In our previous study, we reported an LA fermentation-mediated improvement in the level of soluble Fe (up to 30-fold; Bergqvist *et al.* 2005b). After the simulated gastrointestinal digestion, there was still a 1.5-fold increase in soluble Fe as a result of LA fermentation. The difference in the extent of improvement was expected owing to the reduced solubility of  $\text{Fe}^{3+}$  at the elevated pH (from about 4 to 6) after digestion of the fermented juice. Nevertheless, after *in vitro* digestion, LA fermentation still yielded about a 50–70% strain-specific improvement in the level of soluble Fe compared with fresh juice. This could be, at least partially, explained by the different composition of organic acids between the two fermentations, which may result in different chelating properties while forming soluble Fe complexes.

In contrast to that of other minerals, the availability of Fe is also related to its valence states. In the present study, an

increased level of soluble  $\text{Fe}^{2+}$  in carrot juice was demonstrated after fermentation (15–17-fold), and this increase remained after *in vitro* digestion, although to a lesser extent (4–5-fold). The difference in the degree of enhancement could be caused by the difference in pH before and after digestion (about 4 and 6, respectively) as the acid environment favours the  $\text{Fe}^{2+}$  rather than the  $\text{Fe}^{3+}$  state. This could also explain the increased and then declining content of soluble  $\text{Fe}^{2+}$  in fresh juice after gastric and intestinal digestion, respectively.

However, despite the pH-mediated influence on soluble  $\text{Fe}^{2+}$ , explanations for the remaining higher level of soluble  $\text{Fe}^{2+}$  in the fermented juice after simulated digestion are still missing. This could be explained by the reduction of  $\text{Fe}^{3+}$  and/or by preventing  $\text{Fe}^{2+}$  oxidation as a result of fermentation. Nevertheless, both mechanisms of antioxidative activity could be derived from either LAB or from some carrot-originated antioxidants formed or stimulated during fermentation. Antioxidative activities of LAB have only recently been documented. Lin & Yen (1999) revealed a strain-specific Fe reduction activity in the intracellular cell extract from most of the LAB strains tested. The increased  $\text{Fe}^{2+}$  in carrot juice during fermentation may thus result from intracellular reducing agents released from lysed LAB cells. However, whether the intact LAB cells also have reducing capability remains unclear.

Furthermore, both intact cells and cell extracts from LAB have been reported to prevent  $\text{Fe}^{2+}$ -generated oxidative stress by their chelating activity, which was a strain-specific event (Lin & Yen, 1999; Lee *et al.* 2005). This, does not, however seem to apply to the present findings on the non-strain-specific elevation of the soluble  $\text{Fe}^{2+}$  content of fermented juice even after digestion. The present data support the findings of Ito *et al.* (2003) that the antioxidative activity by a strain of *Streptococcus thermophilus* was not a consequence of  $\text{Fe}^{2+}$  removal from the reaction system of lipid peroxidation. In addition, the increase in  $\text{Fe}^{2+}$  in fermented juice could also be explained in terms of the liberation of  $\text{Fe}^{2+}$  from its food complexes through the action of some degrading enzymes derived from either LAB or carrot. Nevertheless, since the soluble  $\text{Fe}^{2+}$  remained in its reduced state in the presence of  $\text{O}_2$  during fermentation and *in vitro* digestion, some antioxidants are probably involved in preventing the oxidation of  $\text{Fe}^{2+}$ . Further studies are needed to identify such antioxidative agents derived from either LAB or carrot endogenously.

Unlike their effect on total Fe availability, *L. pentosus* FSC1 and *Ln. mesenteroides* FSC2 fermentation mediated a non-strain-specific increase (about 4-fold) in the efficiency of cellular Fe uptake from carrot juice. Noticeably, the degree of fermentation-mediated improvement in the efficiency of cellular Fe uptake and  $\text{Fe}^{2+}$  solubility was similar after *in vitro* digestion, and none of the events was strain-dependent. The improved efficiency of Fe uptake by Caco-2 cells was more likely to be a result of the increase in  $\text{Fe}^{2+}$  availability. Fe absorption has also been reported to be affected by its valence state (Alvarez-Hernandez *et al.* 1991; Nunez *et al.* 1994). In fact, Fe reduction has been reported to be necessary for its absorption by enterocytes (Wollenberg & Rummel, 1987; Han *et al.* 1995). Nevertheless, *Ln. mesenteroides* FSC2 fermentation still yielded a 20% higher level of Fe absorbed by Caco-2 cells than was seen with *L. pentosus* FSC1 (22.7 v. 19.2  $\mu\text{g/l}$  juice per mg protein).

In addition, the uptake of Fe has been shown to be limited by other metals such as Mn, Cu and Zn owing to competition for the intestinal uptake protein divalent metal transporter-1 (Gunshin *et al.* 1997; Garrick *et al.* 2003; Yeung *et al.* 2005). The modest increase in the level of available Mn and Zn in the present study was, however, not expected to influence the uptake of Fe by Caco-2 cells as a result of LA fermentation. Interestingly, the fermentation-stimulated uptake of Fe by Caco-2 cells was retained intracellularly within 1 h of incubation, as indicated by the unaffected transepithelial transfer across the cell monolayer. This could be a result of the cellular regulation of downstream Fe transport, which was suggested to reflect the need for Fe from the basolateral side of cells (Wood & Han, 1998), and the Fe status of cells (Alvarez-Hernandez *et al.* 1991; Arredondo *et al.* 1997). Both parameters were steady in our experimental setting. However, the Caco-2 cell model for studying Fe transepithelial transfer needs further evaluation (Gangloff *et al.* 1996).

To conclude, the influence of LA fermentation on Fe, but not Mn, Zn and Cu, solubility under simulated physiological conditions was related to the strain used. The LA fermentation-enhanced Fe uptake by Caco-2 cells from carrot juice was attributed to the increase in not only Fe availability, but also the efficiency of cellular Fe uptake. The efficiency of transepithelial transfer of Fe across the cells was not, however, influenced by LA fermentation. The enhanced efficiency of Fe uptake resulting from LA fermentation was probably due to the increase in level of available Fe<sup>2+</sup> rather than being a strain-specific event.

LA fermentation is a means of improving the shelf-life of juice without adding preservatives. An evaluation of its organo-oleptic properties alone and in mixtures with other juices needs to be performed. Furthermore, this processing can contribute to an added nutritional value of the product apart from the increased Fe bioavailability, probably seeing other health-benefitting effects such as an attenuation of the postprandial blood glucose and insulin response (Torsdottir *et al.* 1992) and probiotic effects. According to the pool concept, it is likely that this fermented juice, consumed with a meal, will improve Fe bioavailability from the whole meal. So far it is not known whether an effect similar to that found in this study would also be observed with other LA-fermented beverages. This will be investigated further.

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### References

- Alasalvar C, Grigor JM, Zhang D, Quantick PC & Shahidi F (2001) Comparison of volatiles, phenolics, sugars, antioxidant vitamins, and sensory quality of different colored carrot varieties. *J Agric Food Chem* **49**, 1410–1416.
- Alvarez-Hernandez X, Nichols GM & Glass J (1991) Caco-2 cell line: a system for studying intestinal iron transport across epithelial cell monolayers. *Biochim Biophys Acta* **18**, 205–208.
- Archibald F (1986) Manganese: its acquisition by and function in the lactic acid bacteria. *Crit Rev Microbiol* **13**, 63–109.
- Arredondo M, Orellana A, Garate MA & Nunez MT (1997) Intracellular iron regulates iron absorption and IRP activity in intestinal epithelial (Caco-2) cells. *Am J Physiol* **273**, G275–G280.
- Au AP & Reddy MB (2000) Caco-2 cells can be used to assess human iron bioavailability from a semipurified meal. *J Nutr* **130**, 1329–1334.
- Avron M (1963) A sensitive and simple method for determination of ferrocyanide. *Anal Biochem* **6**, 549–554.
- Beard JL, Dawson H & Pinero DJ (1996) Iron metabolism: a comprehensive review. *Nutr Rev* **54**, 295–317.
- Bergqvist SW, Sandberg AS, Andlid T & Wessling-Resnick M (2005a) Lactic acid decreases Fe(II) and Fe(III) retention but increases Fe(III) transepithelial transfer by Caco-2 cells. *J Agric Food Chem* **53**, 6919–6923.
- Bergqvist SW, Sandberg A-S, Carlsson N-G & Andlid T (2005b) Improved iron solubility in carrot juice fermented by homo- and hetero-fermentative lactic acid bacteria. *Food Microbiology* **22**, 53–61.
- Brune M, Hallberg L & Skånberg A-B (1991) Determination of iron-binding phenolic groups in foods. *J Food Sci* **56**, 128–131, 167.
- Cowart RE, Singleton FL & Hind JS (1993) A comparison of bathophenanthrolinedisulfonic acid and ferrozine as chelators of iron(II) in reduction reactions. *Anal Biochem* **211**, 151–155.
- Fernandez M, Sun D, Tosca M & Mc-Namara D (1995) Guar gum effects on plasma low-density lipoprotein and hepatic cholesterol metabolism in guinea pigs fed low- and high-cholesterol diets: a dose-response study. *Am J Clin Nutr* **61**, 127–134.
- Fredrikson M, Carlsson NG, Almgren A & Sandberg AS (2002) Simultaneous and sensitive analysis of Cu, Ni, Zn, Co, Mn, and Fe in food and biological samples by ion chromatography. *J Agric Food Chem* **50**, 59–65.
- Gangloff MB, Lai C, Van Campen DR, Miller DD, Norvell WA & Glahn RP (1996) Ferrous iron uptake but not transfer is down-regulated in Caco-2 cells grown in high iron serum-free medium. *J Nutr* **126**, 3118–3127.
- Garcia-Casal MN, Leets I & Layrisse M (2000) Beta-carotene and inhibitors of iron absorption modify iron uptake by Caco-2 cells. *J Nutr* **130**, 5–9.
- Garrick MD, Dolan KG, Horbinski C, *et al.* (2003) DMT1: a mammalian transporter for multiple metals. *Biometals* **16**, 41–54.
- Gillooly M, Bothwell TH, Torrance JD, MacPhail AP, Derman DP, Bezwoda WR, Mills W, Charlton RW & Mayet F (1983) The effects of organic acids, phytates and polyphenols on the absorption of iron from vegetables. *Br J Nutr* **49**, 331–342.
- Glahn RP, Lai C, Hsu J, Thompson JF, Guo M & Van Campen DR (1998) Decreased citrate improves iron availability from infant formula: application of an in vitro digestion/Caco-2 cell culture model. *J Nutr* **128**, 257–264.
- Gunshin H, Mackenzie B, Berger UV, Gunshin Y, Romero MF, Boron WF, Nussberger S, Gollan JL & Hediger MA (1997) Cloning and characterization of a mammalian proton-coupled metal-ion transporter. *Nature* **388**, 482–488.
- Hallberg L, Brune M & Rossander L (1989) The role of vitamin C in iron absorption. *Int J Vitam Nutr Res Suppl* **30**, 103–108.
- Han O, Failla ML, Hill AD, Morris ER & Smith JC Jr (1994) Inositol phosphates inhibit uptake and transport of iron and zinc by a human intestinal cell line. *J Nutr* **124**, 580–587.
- Han O, Failla ML, Hill AD, Morris ER & Smith JC Jr (1995) Reduction of Fe(III) is required for uptake of nonheme iron by Caco-2 cells. *J Nutr* **125**, 1291–1299.
- Hurrell RF, Reddy M & Cook JD (1999) Inhibition of non-haem iron absorption in man by polyphenolic-containing beverages. *Br J Nutr* **81**, 289–295.
- Ito M, Ohishi K, Yoshida Y, Yokoi W & Sawada H (2003) Antioxidative effects of lactic acid bacteria on the colonic mucosa of iron-overloaded mice. *J Agric Food Chem* **51**, 4456–4460.

- Lee J, Hwang KT, Heo MS, Lee JH & Park KY (2005) Resistance of *Lactobacillus plantarum* KCTC 3099 from kimchi to oxidative stress. *J Med Food* **8**, 299–304.
- Lin M-Y & Yen C-L (1999) Antioxidative ability of lactic acid bacteria. *J Agric Food Chem* **47**, 1460–1466.
- Mazur A, Remesy C, Gueux E, Levrat MA & Demigne C (1990) Effects of diets rich in fermentable carbohydrates on plasma lipoprotein levels and on lipoprotein catabolism in rats. *J Nutr* **120**, 1037–1045.
- Miller NJ, Sampson J, Candeias LP, Bramley PM & Rice-Evans CA (1996) Antioxidant activities of carotenes and xanthophylls. *FEBS Lett* **384**, 240–242.
- Narasinga Rao BS & Subba Rao K (1992) Studies on the role of iron binding ligands and the intestinal brush border receptors in iron absorption. *Indian J Biochem* **29**, 214–218.
- National Food Administration (2002) The NORBAGREEN 2002 study: Consumption of vegetables, potatoes, fruit, bread and fish in the Nordic and Baltic countries. *TemaNord* **2003**, 556.
- Nunez MT, Alvarez X, Smith M, Tapia V & Glass J (1994) Role of redox systems on Fe<sup>3+</sup> uptake by transformed human intestinal epithelial (Caco-2) cells. *Am J Physiol* **267**, C1582–C1588.
- Pandey A, Bringel F & Meyer J (1994) Iron requirement and search for siderophores in lactic acid bacteria. *Appl Microbiol Biotechnol* **40**, 735–739.
- Rice-Evans CA, Miller NJ & Paganga G (1996) Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radic Biol Med* **20**, 933–956.
- Stiles ME (1996) Biopreservation by lactic acid bacteria. *Antonie Van Leeuwenhoek* **70**, 331–345.
- Svanberg U, Lorri W & Sandberg A-S (1993) Lactic fermentation of non-tannin and high-tannin cereals: effects on in vitro estimation of iron availability and phytate hydrolysis. *J Food Sci* **58**, 408–412.
- Teucher B, Olivares M & Cori H (2004) Enhancers of iron absorption: ascorbic acid and other organic acids. *Int J Vitam Nutr Res* **74**, 403–419.
- Torsdottir I, Alpsten M, Holm G, Sandberg A-S & Tölli J (1991) A small dose of soluble alginate-fiber affects postprandial glycemia and gastric emptying in humans with diabetes. *J Nutr* **121**, 795–799.
- Torsdottir I, Blomqvist R, Ekman R, Hagander B & Sandberg A-S (1992) Fermented or fresh vegetables decrease the postprandial blood glucose and insulin levels in health persons. *Scand J Nutr* **36**, 4–7.
- Umbreit J (2005) Iron deficiency: a concise review. *Am J Hematol* **78**, 225–231.
- Wollenberg P & Rummel W (1987) Dependence of intestinal iron absorption on the valency state of iron. *Naunyn Schmiedeberg Arch Pharmacol* **336**, 578–582.
- Wood RJ & Han O (1998) Recently identified molecular aspects of intestinal iron absorption. *J Nutr* **128**, 1841–1844.
- Yeung CK, Glahn RP & Miller DD (2005) Inhibition of iron uptake from iron salts and chelates by divalent metal cations in intestinal epithelial cells. *J Agric Food Chem* **53**, 132–136.