Iron, catechin and ferulic acid inhibit cellular uptake of β-carotene by reducing micellization

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J.K. designed the study and conducted experiments, collected data and wrote the manuscript; W.S. provided methods and comments; J.F. was involved with the study design and provided comments.

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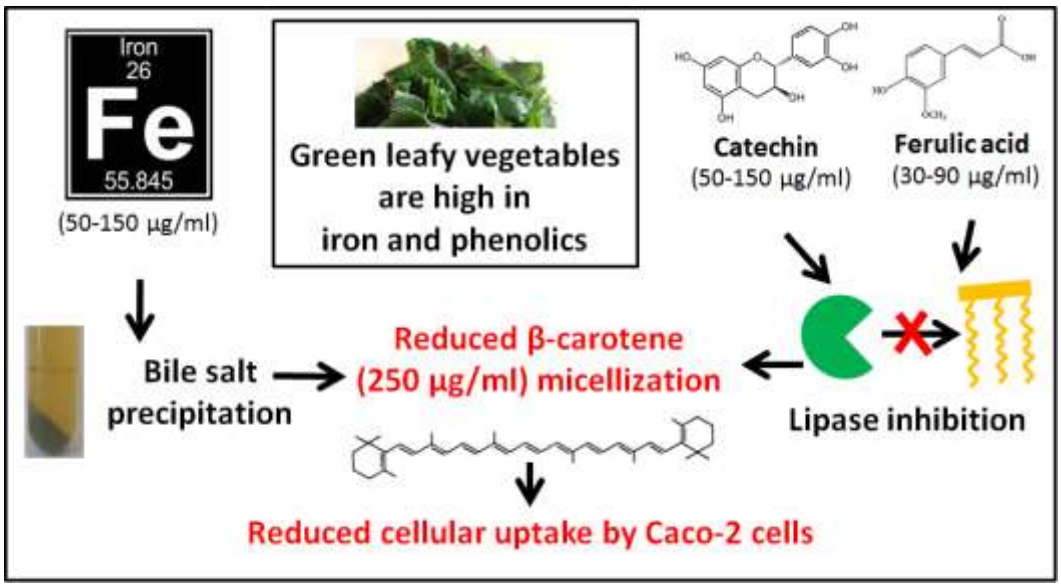

Abstract

Green leafy vegetables have low β-carotene bioavailability, which we hypothesised to be, at least in part, due to high contents of fibre, minerals, and phenolics. We investigated the effects of pectin (40-120 μg/mL), iron (50-150 μg/mL), ferulic acid (30-90 μg/mL) and catechin (50-150 μg/mL), in a model system, on β-carotene micellization (*in vitro* digestion) and intestinal absorption (Caco-2 cell model). Iron, pectin, ferulic acid and catechin on average, reduced ($p<0.05$) β-carotene micellization (1.49±0.05 μmol/L) by 66.9, 59.3, 43.2 and 51.7%, respectively. Iron reduced micellization by precipitating bile salts from solution and ferulic acid and catechin by inhibition of pancreatic lipase. β-carotene uptake by Caco-2 cells (2.63±0.22%) was reduced ($p<0.05$) by 37.4, 70.1, 77.0 and 75.1%, respectively, when digested with pectin, iron, ferulic acid or catechin. However, when individual test compounds were added to already micellized β-carotene, they did not inhibit β-carotene uptake. The large reductions in β-carotene micellization observed *in vitro*, warrant further investigation in humans using model green leafy vegetable systems to elucidate their relevance under real-life conditions.

Key words: β-carotene, bioavailability, dietary inhibitors, green leafy vegetables, pectin,

Chemical compounds studied in this article:

Pectin (PubChem CID: 441476); Iron sulphate (PubChem CID: 24393); ferulic acid (PubChem CID: 445858); naringenin (PubChem CID: 932); catechin (PubChem CID: 9064); β-carotene (PubChem CID: 5280489)

1. Introduction

While the prevalence of vitamin A deficiency in 138 low/middle-income countries decreased from 39% in 1991 to 29% in 2013, it increased from 45% to 48% in sub-Saharan Africa.[1] This occurred despite the implementation of high-dose vitamin A supplementation throughout the region. High-dose vitamin A treatment only has a minor, short-lived impact on the prevalence of sub-clinical and mild to moderate vitamin A deficiency[2] and a higher dietary intake of vitamin A and pro-vitamin A-rich foods is recommended to prevent it.[1-6]

In low socio-economic populations in sub-Saharan Africa, where vitamin A deficiency is prevalent, pro-vitamin A-rich plant foods, such as green leafy vegetables, are the most important sources of vitamin A. Consumption of green leafy vegetables, however, often does not improve vitamin A status[7, 8] or only to a smaller extent compared to other pro-vitamin A-rich plant foods, such as carrots and orange-fleshed sweet potato.[5, 9] This is due to the low oral bioavailability of pro-vitamin A, most importantly β-carotene, from green leafy vegetables compared to other pro-vitamin A-rich plant foods.[10] Very little research has been done investigating the cause of the low bioavailability of β-carotene from green leafy vegetables and no single cause has been identified. One explanation is that the carotenoids in the chromoplasts of green leafy vegetables are complexed to proteins, which are much less bioaccessible during digestion, compared to the crystalline forms in carrots and tomatoes and being dissolved in oil droplets in pumpkin.[11] On the other hand, green leafy vegetables also contain much more dietary fibre, iron and phenolic compounds than other provitamin A rich crops mentioned above.[11]

The matrix effects of dietary lipids, fibre and food processing on β-carotene bioavailability has been studied extensively.[12] Fibre is a widely recognised inhibitor of β-

carotene bioavailability, of which pectin has been found to have the highest inhibitory effect.[13] It is however, only recently that the effects of minerals and phenolic compounds on carotenoid bioavailability have been considered.[12]

Divalent minerals and phenolic compounds have been found to inhibit the micellization and Caco-2 uptake of carotenoids. Iron, zinc and calcium, at supplemental levels, have been found to decrease the micellization[15,16] and Caco-2 uptake[15,17,18] of carotenoids. Borel et al.[19] and Corte-Real et al.[20] evaluated the effect of calcium on carotenoid bioavailability in *in vivo*/human studies Borel et al.[19] found that a calcium supplement (500 mg) reduced lycopene bioavailability (from tomatoes) with 83%, while Corte-Real et al.[20] found calcium supplements (500-1000 mg) had no effect on carotenoid bioavailability from spinach. Corte-Real et al.[20] attributed the contrasting results in these two studies to differences in the food matrixes. Catechin and ferulic acid are two of the most abundant phenolics in plant products also present in green leafy vegetables.[21,22] The effect of ferulic acid, catechin and iron (in molar ratios found in green leafy vegetables) on β-carotene bioavailability has not been investigated.

Divalent minerals and phenolics have been found to both, interact with pectin and also have an effect on β-carotene bioavailability. However, the effects of pectin in combination with iron and phenolics on the bioavailability of β-carotene have not been evaluated in any study, be it *in vitro* or *in vivo*. Importantly, this study will provide the first information on the interaction between divalent minerals/phenolics with pectin and the resulting effect on β-carotene bioaccessibility.

This study aimed to evaluate, in a model system, the effect of the dietary fibre pectin, the phenolic compounds catechin, ferulic acid and naringenin, and the mineral iron, alone and in combination, on β-carotene bioaccessibility and uptake into Caco-2 cells.

2. Materials and methods

2.1. Chemicals

Apple pectin (with 50-75% esterification; 93854), ferulic acid (≥99%), naringenin (≥95%), catechin (≥99%), porcine pancreatic lipase (L3126), porcine pancreatin (P3292), porcine bile extract (B8631), Dulbecco's Modified Eagle Medium, *p*-Nitrophenyl palmitate and β-carotene (22040) were all obtained from Sigma Aldrich (Steinheim, Germany). Iron sulphate ($FeSO_4.7H_20$, ≥99.5%), sodium pyruvate and non-essential-amino-acids were obtained from Carl Roth (Karlsruhe, Germany). Foetal bovine serum (non-heat-treated) were obtained from Thermo Fisher (Karlsruhe, Germany), penicillin and streptomycin from Merck (Darmstadt, Germany) and the 9-cis- and 13-cis-β-carotene standards form CaroteNature GmbH (Münsingen, Switzerland)

2.2. Experimental design

The β-carotene used in the digestion model was composed of approximately 83.0±1.8% trans-, 5.80±0.82% 9-cis- and 11.2±1.4% 13-cis-β-carotene, respectively (as analysed by HPLC; section 2.5). While unsaturated fatty acids in general improve β-carotene micellization[23], olive oil was used as lipid source as is contains less ferulic acid than other seed oils, such as flaxseed and soybean.[24] The experimental design to estimate (*in vitro*) the effect of fibre (apple pectin, iron, ferulic acid, catechin and naringenin on β-carotene bioavailability is displayed in **Figure 1**. Naringenin was included as a positive control, as it was previously reported to inhibit the uptake of lutein into Caco-2 cells.[25] The co-

consumption of β-carotene and naringenin in rats also led to significantly lower concentrations of retinoids in the liver and intestines, compared to β-carotene consumed alone.[26]

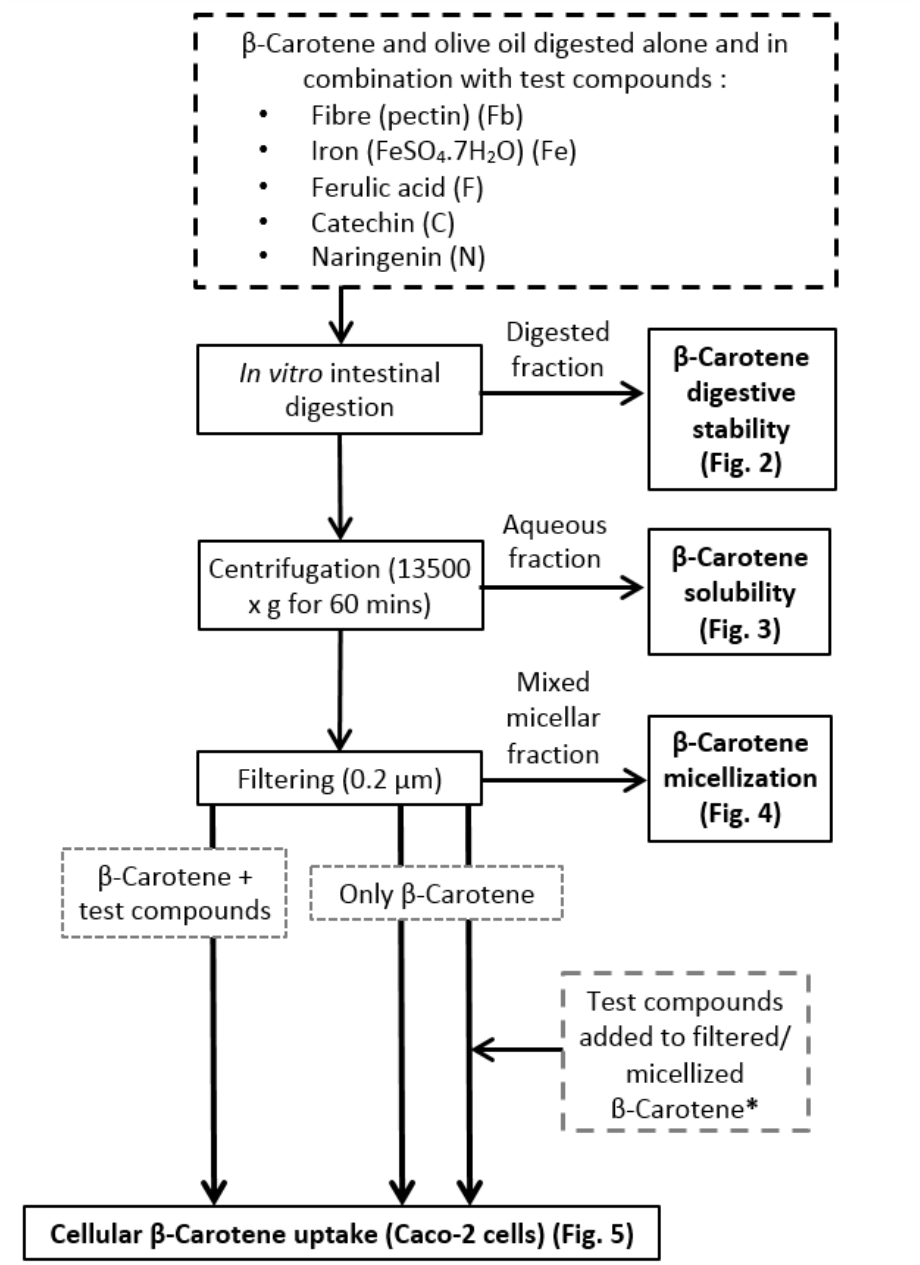

Figure 1: Experimental design to determine the effect of fibre (Fb), iron (Fe), ferulic acid (F), catechin (C), and naringenin (N) on the *in vitro* β-carotene digestive stability, solubility, micellization and uptake by Caco-2 cells. * - Evaluated the effect of test compounds only on the specific β-carotene absorption by Caco-2 cells and not on the digestive processes (solubility/micellization).

The concentrations of the test compounds were calculated according to the nutrient content of various green leafy vegetables. The average β-carotene and iron contents from a variety of green leafy vegetables (n>13)[12] were 32.3 and 18.9 mg/100 g (db). When the dried green leafy vegetable was added to the digestion at 0.75 g/mL, the final concentrations of β-carotene and iron were approximately 250 and 150 µg/mL, respectively (**Table 1**). Ferulic acid, catechin and naringenin were added, at the highest concentration (C3), at a 1:1 molar ratio to β-carotene.

Table 1: Concentrations (µg/ml digest) and combinations of β-carotene and olive oil with fibre (Fb), iron (Fe), ferulic acid (F), catechin (C), and naringenin (N) subjected to *in vitro* intestinal digestion

***Control: β-carotene (250 µg / per ml digest) & olive oil (100 µl / per ml digest)**		**Fb**	**Fe**	**F**	**C**	**N**
		µg/ml digest				
+ Fibre (Fb)	**1**	40				
	2	80				
	3	120				
+ Iron ($FeSO_4.7H_2O$) **(Fe)**	**1**		50			
	2		100			
	3		150			
+ Ferulic acid (F)	**1**			30		
	2			60		
	3			90		
+ Catechin (C)	**1**				50	
	2				100	
	3				150	
+ Naringenin (N)	**1**					45
	2					90
	3					135
+ Fb & Fe		80	100			
+ Fb & F		80		60		
+ Fb & C		80			100	
+ Fb & N		80				90
+ F & C				60	100	
+ Fb & F & C		80		60	100	
+ F & C & N				60	100	90
+ Fb & F & C & N		80		60	100	90

***-The control digestion (final volume of 20 ml) contained only β-carotene and olive oil. The effects of all subsequent inhibitors were tested by addition to the control digestion.**

2.3. β-Carotene bioaccessibility (digestive stability, solubilisation, micellization)

The *in vitro* simulation of small intestinal digestion (**Figure 1**) was performed as previously described[23] with some minor modifications. Only an intestinal digestion was used as the model system did not include the matrix effect of whole foods and the β-carotene was already solubilised into the oil phase and it has been previously shown that gastric digestion does not play a significant role in the solubilisation of carotenoids into the aqueous phase.[27]

For addition of the β-Carotene to the digestion, it was dissolved in hexane and added to the digestion tubes. The hexane was evaporated under vacuum, and the residue dissolved in the olive oil, after which the other test compounds were added. For each digestion (final volume of 20 mL), β-carotene (250 µg/mL), olive oil (100 µL/mL) and various concentrations and combinations of the test compounds (Table 1) were digested with porcine pancreatin (0.8 mg/mL), porcine pancreatic lipase (1.6 mg/mL) and porcine bile extract (3.6 mg/mL). The pH of the digestion was adjusted to 7, topped with nitrogen gas, and incubated for 2 h at 37 °C, with vigorous shaking for 5 s every 20 min to ensure emulsion droplet formation and lipase-substrate interface-interaction. A sub-sample of the digest was collected and analysed for β-carotene to determine the effect of the test compounds on the digestive stability of β-carotene ($\frac{\beta-carotene\ in\ digesta}{\beta-carotene\ in\ digesta\ of\ control}\ x\ 100$) throughout the simulated small intestinal digestion.

The digested samples were then centrifuged (13500 × g, 60 min, 4 °C) and a sub-sample of the supernatant collected and analysed for solubilised β-carotene in order to determine the effect of the test compounds on β-carotene solubility

($\frac{\beta-carotene\ in\ soluble\ fraction}{Total\ \beta-carotene\ digested}\ x\ 100$). The soluble fraction would include bile salt-lipid micelles as well as larger undigested lipid emulsions which are less physiologically relevant to β-carotene absorption. The remainder of the soluble fraction was filtered (Filtropur S, 0.2 µm, Sarstedt, Nümbrecht, Germany) to separate bile salt-lipid mixed micelles from the larger lipid emulsions.

The filtered micellar fraction was analysed for β-carotene to determine the effect of the test compounds on β-carotene micellization ($\frac{\beta-carotene\ in\ filtered\ micellar\ fraction}{Total\ \beta-carotene\ digested}\ x\ 100$). All collected samples were topped with nitrogen gas and stored at -80 °C for a maximum of one week prior to HPLC analysis.

2.4. β-Carotene uptake into Caco-2 cells

The intestinal absorption of β-carotene was simulated using differentiated Caco-2 cells (14-16 days post-confluence) as previously reported.[28] Caco-2 cells (HTB-37, ATCC, Wessel, Germany) were cultured in Dulbecco's Modified Eagle Medium (Sigma Aldrich) containing 10% foetal bovine serum, 1% sodium pyruvate, 1% non-essential-amino-acids (Carl Roth) and 1% penicillin and streptomycin. Cells were seeded in 12-well culture plates at 6×10^4 cells per well and all experiments conducted between passages 12 and 20. The differentiation of each passage was monitored using a Transwell membrane insert, to ensure TEER of 250 Ω cm^2 before experiments were conducted.

Differentiated Caco-2 cells were rinsed with phosphate-buffered saline and then the micellar fraction and serum-free Dulbecco's Modified Eagle Medium (with 1% antibiotics) were added at a ratio of 1:3 (v/v), and cells incubated for 4 h (**Supplementary Figure S1**).

The highest concentration (that of the control) of β-carotene added to the cells was 0.47 µmol/L, well below the absorption saturation point (>10 µmol/L) of β-carotene for Caco-2 cells.[29] Samples were randomly distributed between the different wells and plates. After the 4 h incubation, the cell culture supernatant was removed and discarded and the cells were rinsed twice with phosphate-buffered saline. Cells were detached using 0.5 M NaOH, covered with nitrogen gas, and stored at -80 °C for a maximum of 48 h prior HPLC analysis.

2.5. Quantification of β-carotene by HPLC

Sample preparation and saponification[30] and β-carotene extraction[31] were performed as previously described. Briefly, all samples (0.5 mL) were mixed with 1% ascorbic acid (2 mL) in ethanol including the internal standard (β-apo-8'-carotenal-methyloxime) (100 µL of 12 µg/100 mL) and saturated potassium hydroxide (300 µL of 53 mg/ 100 mL) for saponification under shaking conditions in a light-protected water bath (2 h, at 37 °C). Samples were cooled on ice, neutralized with glacial acetic acid, and butylated hydroxytoluene in ethanol (1 mg/mL) added. The samples were then extracted twice with hexane, which was evaporated in a centrifugal vacuum concentrator. The final extract was reconstituted in ethanol: acetonitrile (1:3 v/v) and analysed on an HPLC system comprised of a Merck Hitachi HPLC (L-6200 intelligent pump, AS-2000A auto sampler, and L-5025 column thermostat) equipped with a UV-detector (Jasco UV-975) operated at a wavelength of 450 nm. Data were acquired, processed, and evaluated with the Clarity chromatographic station (Data Apex Ltd., Prague, Czech Republic). Standards of 9-cis- and 13-cis-β-carotene were used to confirm peak retention times and the concentrations of the cis-isomers were calculated using the external all-trans β-carotene standard.

2.6. Pancreatic lipase activity and bile salt precipitation

The effect of apple pectin (80 μg/mL), iron (100 μg/mL), ferulic acid (60 μg/mL), catechin (100 μg/mL) and naringenin (90 μg/mL) on porcine pancreatic lipase (12 mg/mL) activity was measured with a chromogenic assay.[32] *p*-Nitrophenyl palmitate (150 mmol/L) was used as substrate and the lipase activity measured over 30 min and the slopes of the resulting linear curves compared.

The effect of iron on the precipitation of bile salts was measured by adding $FeSO_4.7H_20$ to a bile solution in the same molar ratio as added during the digestions. The mixtures were vortexed for 10 s and centrifuged at 150 × g for 5 min and the amount of precipitate visually evaluated.

2.7. Statistical analysis

Results are given as arithmetic mean (n=4) with standard deviation and all statistical analysis was done using GraphPad Prism (GraphPad Software, La Jolla, CA). Normality of data and equality of variance were assessed using the Shapiro-Wilk and Levene's tests, respectively. One-way analysis of variance (ANOVA) with Tukey's HSD post-hoc test was used to test for significant differences between individual means. The significant differences between the slopes of the Pearson's regressions from the lipase activity data were calculated using one-way ANOVA with Tukey's HSD post-hoc test. Statistical significance for all tests was considered at a confidence level of 95% ($p<0.05$).

3. Results and discussion

The studied *in vitro* parameters describing β-carotene bioaccessibility, namely digestive stability, solubility, and micellization, as well as the uptake into Caco-2 cells each provide

insights into specific aspects of how the test compounds may affect β-carotene bioavailability *in vivo*. The degradation of β-carotene during *in vitro* intestinal digestion can be increased or decreased by the presence of other test compounds, the extent of which is expressed as β-carotene digestive stability (Section 3.1). Solubility and micellization data (Section 3.2) give insight into how much of the β-carotene present is incorporated into the lipid phase/fat emulsion and mixed micelles, respectively. The uptake into Caco-2 cells simulates the absorption of β-carotene by the enterocytes of the small intestine.

In the present work, the test compounds were either added during β-carotene digestion or afterwards to discern between effects on β-carotene uptake caused by altered micellization and those directly altering absorption into Caco-2 cells. Although trans-, 9-cis- and 13-cis-β-carotene were analysed in all experiments, to simplify data presentation, the results and discussion focus on the more abundant and nutritionally significant all-trans β-carotene (**Figures 2-5**). Data on the bioaccessibility and uptake of 9-cis- and 13-cis-β-carotene are available as supplementary data (**Supplementary Figures S2-S10**).

3.1. β-Carotene digestive stability

Pectin (by 20.2-33.8%), iron (32.5-61.7%), ferulic acid (15.1-36.1%), catechin (42.0-50.3%) and naringenin (7.4-27.9%) all decreased the digestive stability of all-trans β-carotene (**Figure 2A**). While these reductions are high compared to those observed in food systems, Kopec et al.[33] who evaluated in a model system the effect of different sources of iron on β-carotene and lycopene digestive stability, also observed reductions in β-carotene stability of more than 50%.

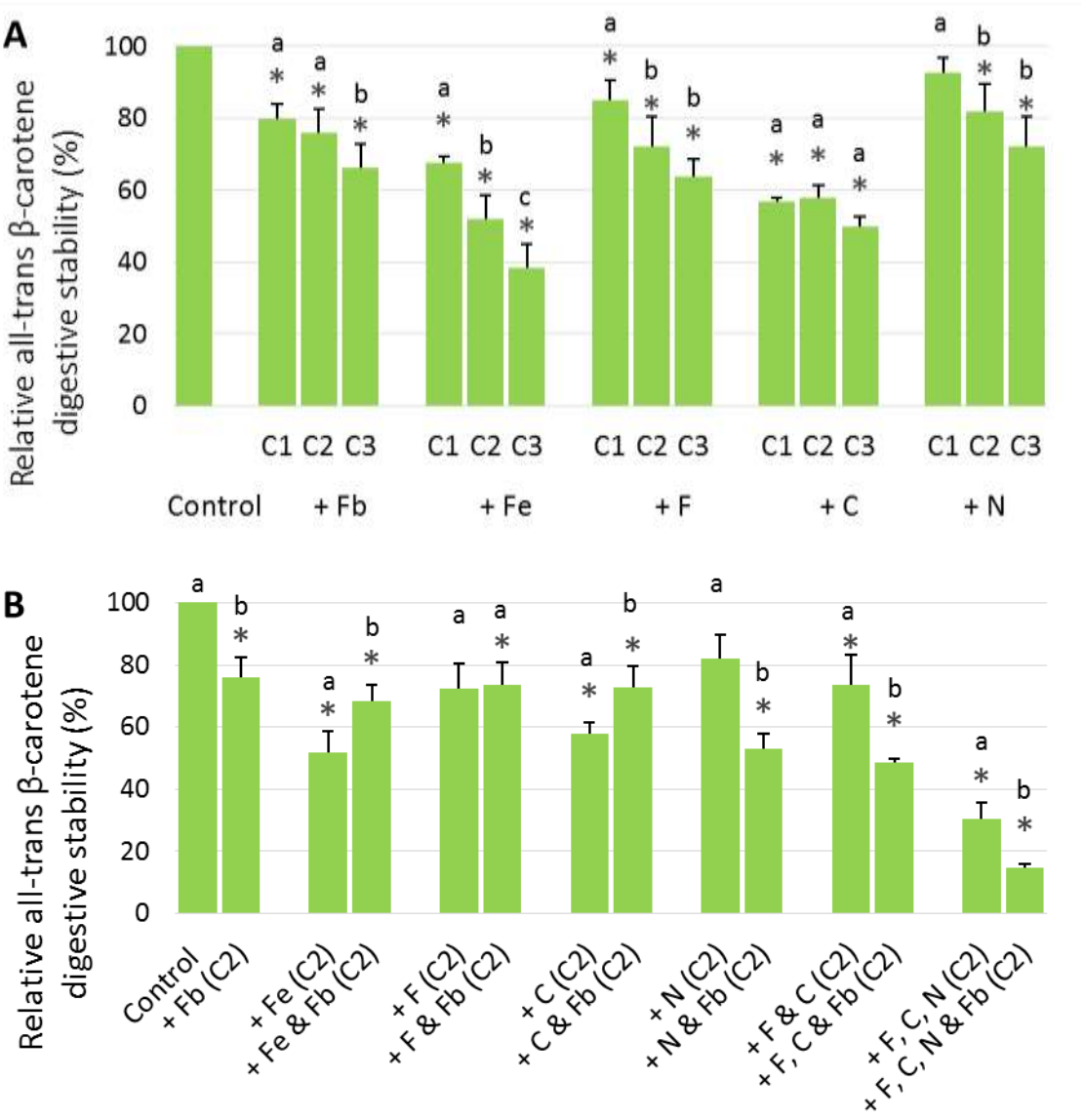

Figure 2: The effects of increasing concentrations (A) and combinations (B) of fibre (Fb), iron (Fe) ferulic acid (F), catechin (C) and naringenin (N) on the intestinal *in vitro* all-trans β-carotene digestive stability (% of control - β -carotene digested alone). C1 is the lowest concentration of Fb (40 mg/ml), Fe (50 mg/ml), F (30 mg/ml), C (50 mg/ml) and N (45 mg/ml) added, with C2 and C3 double and three fold the concentration of C1, respectively. Error bars indicate one standard deviation (n=4). * - Significant difference from the control at p<0.05. abc- bars (of the same test compound) with different letters are statistically different at p<0.05.

Iron, as a single compound, had the largest degradative effect on all-trans β-carotene (**Figure 2A**), which could be due to iron-induced oxidation reactions. Some of the added ferrous iron ($FeSO_4.7H_20$) may have oxidised to ferric iron during the *in vitro*

digestion. Ferric iron reacts with carotenoids to form cation radicals (Car + Fe^{3+} ↔ Car•$^{+}$ + Fe^{2+}), which react with ferric iron to form dications (Car•$^{+}$ + Fe^{3+} → Car^{2+} + Fe^{2+}).[34] Ferric iron also acts as a catalyst of auto-oxidation and thermal degradation of β-carotene.[34] Kopec et al.[33] also found that ferrous iron increased β-carotene oxidation in a dose dependent manner when added at levels up to 10 equivalents, but not when added at equivalents of 39. The authors argued that when ferrous sulphate was present in excess that inert perferryl and ferryl intermediates could possibly form. This agrees with our results where iron was added at equivalents to β-carotene of 5.8.

The all-trans β-carotene digestive stability with both iron + pectin and catechin + pectin was higher compared to digestions with iron or catechin alone (Figure 2B). It is possible that the soluble fibre may have bound some iron[35] and catechin[36] and thus reduced their respective degrading effects on all-trans β-carotene. The presence of pectin in digestions with naringenin, ferulic acid + catechin, and ferulic acid + catechin + naringenin, however, further decreased all-trans β-carotene digestive stability by 29.0, 25.1 and 15.6 percentage points, respectively. The mechanisms by which these compounds, together with pectin, increased β-carotene degradation are unclear and require further investigation.

3.2. β-Carotene solubility and micellization

While the effect of the compounds on the β-carotene solubility (**Figure 3**) varied, all compounds substantially decreased the incorporation of β-carotene into the mixed micelles (**Figure 4**).

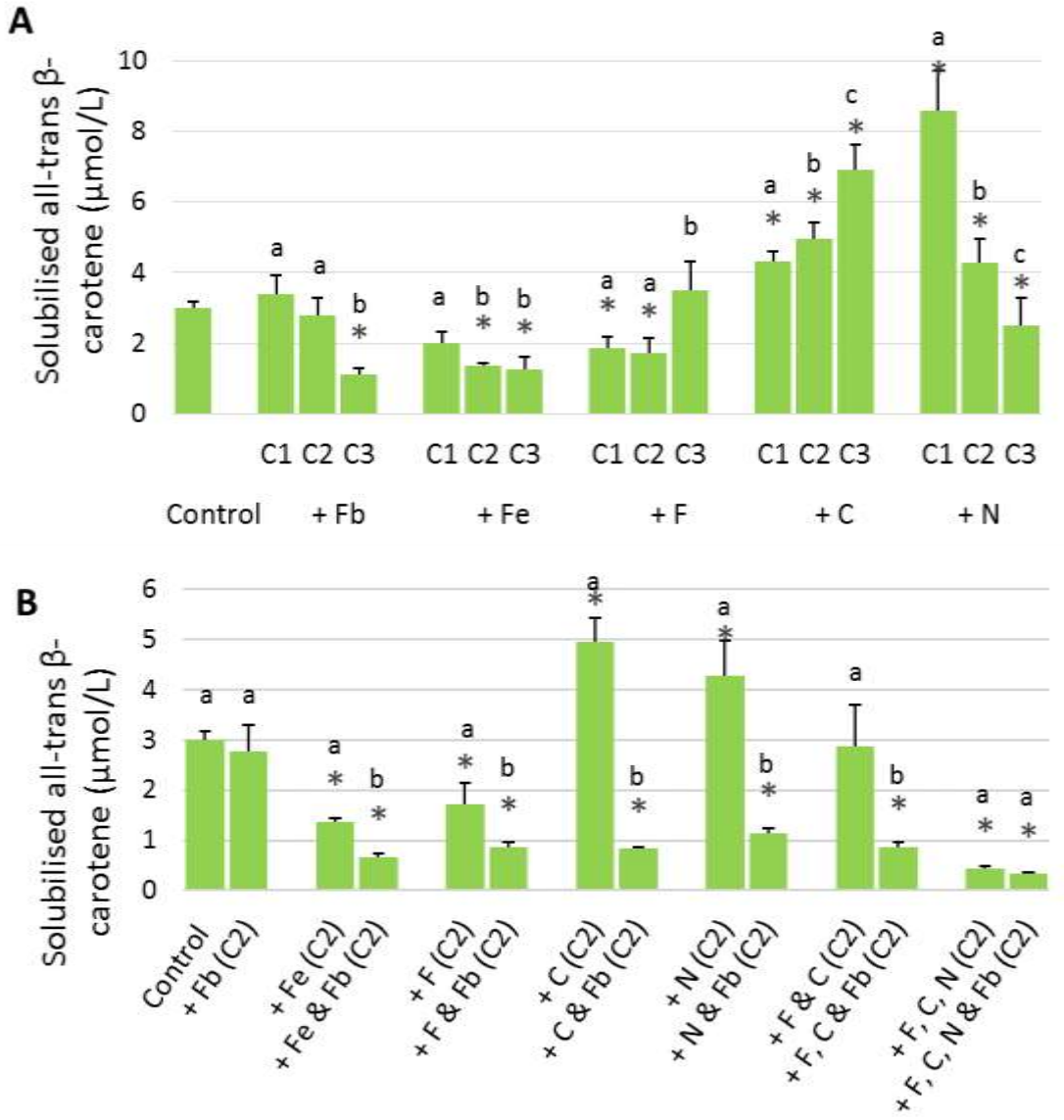

Figure 3: The effects of increasing concentrations (A) and combinations (B) of fibre (Fb), iron (Fe) ferulic acid (F), catechin (C) and naringenin (N) on the intestinal *in vitro* all-trans β-carotene solubility (µmol/L). C1 is the lowest concentration of Fb (40 mg/ml), Fe (50 mg/ml), F (30 mg/ml), C (50 mg/ml) and N (45 mg/ml) added, with C2 and C3 double and three fold the concentration of C1, respectively. Error bars indicate one standard deviation (n=4). * - Significant difference from the control (β -carotene digested alone) at p<0.05. abc- bars (of the same test compound) with different letters are statistically different at p<0.05.

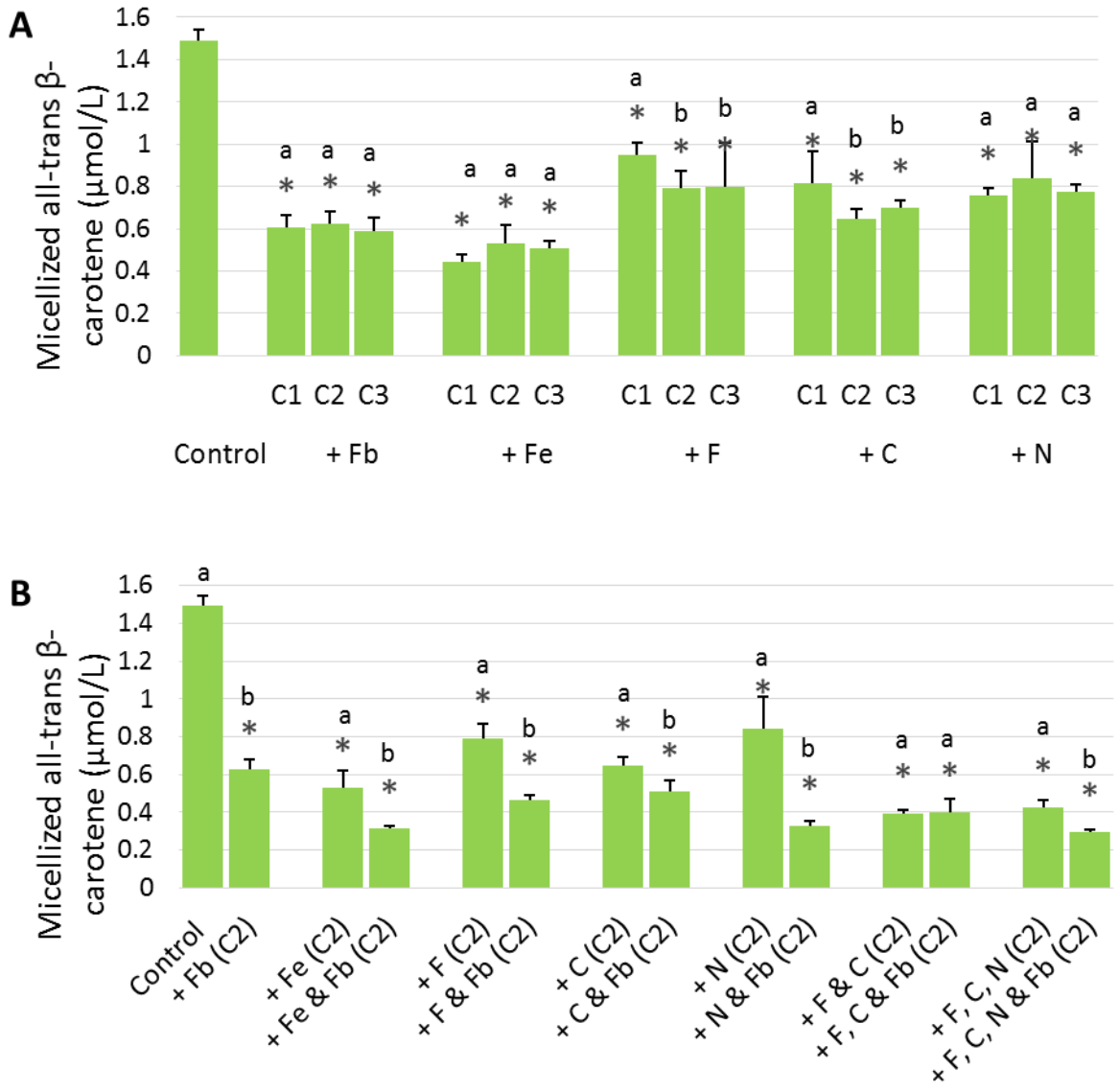

Figure 4: The effects of increasing concentrations (A) and combinations (B) of fibre (Fb), iron (Fe) ferulic acid (F), catechin (C) and naringenin (N) on the intestinal *in vitro* all-trans β-carotene micellization (µmol/L). C1 is the lowest concentration of Fb (40 mg/ml), Fe (50 mg/ml), F (30 mg/ml), C (50 mg/ml) and N (45 mg/ml) added, with C2 and C3 double and three fold the concentration of C1, respectively. Error bars indicate one standard deviation (n=4). * - Significant difference from the control (β -carotene digested alone) at p<0.05. abc- bars (of the same test compound) with different letters are statistically different at p<0.05.

Pectin added at 120 µg/mL, decreased all-trans β-carotene solubility by 63.2% (**Figure 3A**). One would expect pectin, being a water-soluble fibre that can physically entrap β-carotene, to have increased β-carotene solubility in water.[36] The digestive conditions,

however, are not optimal for pectin solubility and it is possible that at the high concentration (120 μg/mL), the centrifugation precipitated some of the pectin out of the aqueous solution (a solid residue was observed after centrifugation). This precipitated pectin would include the entrapped β-carotene, thus reducing the amount of soluble all-trans β-carotene. Digestion with pectin (40-120 μg/mL) substantially reduced micellization of all-trans β-carotene by 58.2-60.5% (**Figure 4A**). This may be due to the entrapment of β-carotene, which was then unavailable for incorporation into mixed micelles, and/or was removed with the pectin during filtration. Pectin has also been found to decrease lipase activity[37], which was not observed in this study (**Supplementary Table S1**). The concentrations of pectin used in this study (40-80 μg/mL) were probably too low to inhibit lipase activity. The extent of lipase inhibition is dependent on the type of lipase (e.g. lingual lipase is more sensitive than pancreatic lipase), concentration of the pectin and the degree of methoxylation[38]. Tsujita et al.[38] found that pectin at 500 μg/mL reduced pancreatic lipase activity with only 10%.

Digestion with iron resulted in the lowest amounts of soluble and micellized all-trans β-carotene (Figures 3A & 4A). It has been proposed that iron may form complexes with bile salts on the micellar surface, thereby compromising micelle stability.[39] More probable, however, is that the iron precipitated the bile salts out of solution, as observed when iron was added to the bile extract (Supplementary Figure S4). Decreased amounts of bile salts in solution would result in a reduction in the formation of micelles. This is in agreement with the precipitation of bile salt in the presence of iron observed by Biehler et al.[19] who proposed that divalent iron forms soaps with free fatty acids and bile salts, resulting in their precipitation.

Interestingly, with increasing concentrations of ferulic acid and catechin, the all-trans β-carotene solubility increased (Figure 3). This could have been due to lipid-partitioning properties of the phenolics and the ability to stabilise the lipid-in-water emulsion. In our study, ferulic acid and catechin were added to the digestions at concentrations of approximately 150, 300 and 450 µmol/L (Table 1). Ferulic acid[40] and catechin,[41] at concentrations of 250 and 200 µmol/L, respectively, have been reported to partition into a nonpolar solution at 6% and 14%, respectively. However, when added to oil-in-water emulsions at a higher concentration of 500 µmol/L, 52% of ferulic acid[42] and 58% of catechin[41] partitioned into a nonpolar oil phase.

Catechin has also been found to increase the lipid: water interfacial area of an olive oil-in-water emulsion by approximately 30%,[42] which was explained by the amphiphilic nature of its chemical structure. However, despite the positive effect of catechin and ferulic acid on all-trans β-carotene solubility, the addition of these compounds to the digestion reduced all-trans β-carotene micellization (**Figure 4A**). Both catechin and ferulic acid was found to inhibit pancreatic lipase activity by 13.6 and 21.3% ($p<0.001$), respectively (**Supplementary Table S1**), which would explain the reduced micellization. Karamać et al.[43] and Ruiz et al.[44] found that ferulic acid and catechin at approximately 8 µmol/L, inhibited lipase activity by 16% and 22%, respectively. Reduced lipase activity would decrease triacylglycerol hydrolysis at the lipid-water interface[45] and subsequently decrease production of fatty acids and monoacylglycerols, resulting in reduced formation of mixed micelles. In line with this, ferulic acid (0.5% of the diet) decreased plasma triacylglycerols and increased faecal lipid excretion in mice fed a high-fat diet (17% w/w).[46]

Naringenin, at all concentrations, decreased the micellization of β-carotene. Poulaert et al.[47] evaluated the effect of grapefruit juice and naringenin on the micellization of β-carotene from orange fleshed sweet potato, and also found that naringenin decreases β-carotene micellization. These[47] and other authors[26] attributed it to competition for inclusion in the micelles. Increasing concentrations of naringenin had no effect on the all-trans β-carotene micellization (**Figure 4A**). Supporting this, naringenin (0.3 µmol/L) also did not inhibit pancreatic lipase activity (**Supplementary Table S1**). Batubara et al.[48] evaluated the effect of naringenin on lipase activity using the 2,3-dimercapto-1-propanol tributyrate method and also found a negligible inhibitory effect with concentrations up to 2 µmol/L.

The addition of pectin to digestions with iron, ferulic acid, catechin, naringenin or mixes of the latter three phenolics further reduced all-trans β-carotene solubility (**Figure 3B**) and micellization (**Figure 4B**).

Pectin, iron, ferulic acid and catechin, all present in high concentrations in green leafy vegetables, inhibit in vitro β-carotene micellization. Mechanisms of inhibition identified in this study include bile salt precipitation (iron) and lipase inhibition (ferulic acid and catechin).

3.3. β-Carotene intestinal uptake by Caco-2 cells

When digested with pectin, iron, ferulic acid, catechin, naringenin and a mix of the latter three phenolics, all-trans β-carotene uptake into Caco-2 cells were 28.5, 34.4, 59.1, 50.2, 42.2 and 69.8% lower, respectively than that of β-carotene digested alone (**Figure 5A**). Fractional uptake (% uptake of amount of β-carotene added to the cells) of all-trans β-carotene from digestions with pectin, iron, ferulic acid, catechin, naringenin and the

combination of ferulic acid + catechin + naringenin were 37.4, 70.1, 77.0, 75.1, 58.4 and 66.4% lower, respectively, than that of β-carotene digested alone (**Figure 5A**).

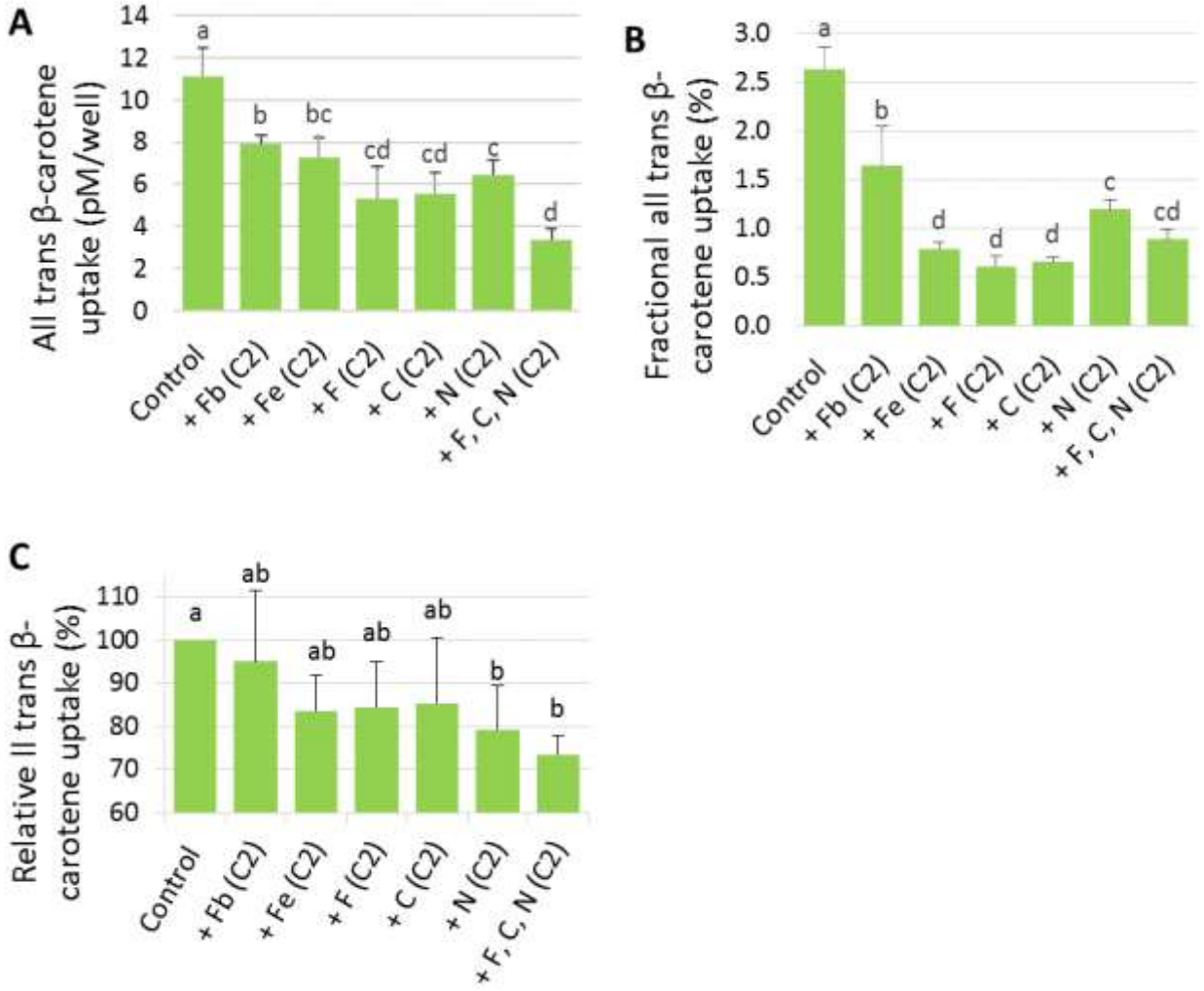

Figure 5: The effects of intestinal *in vitro* digestion of β-carotene with fibre (Fb) (80 mg/ml), iron (Fe) (100 mg/ml), ferulic acid (F) (60 mg/ml), catechin (C) (100 mg/ml) and naringenin (N) (90 mg/ml) on the (A) absolute uptake (amount of β-carotene absorbed by the cells) (pM/well) and (B) fractional uptake (percentage of micellirized β-carotene added to the cells) of all-trans β-carotene by Caco-2 cells. (C) The effect of adding Fb, Fe, F, C and N, after intestinal *in vitro* digestion, to micellised β-carotene, on the all-trans β-carotene uptake by Caco-2 cells (relative (%) to uptake of β-carotene digested alone). Error bars indicate one standard deviation (n=5). abc- bars with different letters are statistically different at p<0.05.

Different theories regarding the underlying mechanisms of the inhibition of the intestinal absorption of carotenoids by dietary fibre,[49,50] iron,[19] and phenolic compounds have been proposed.[26,47,51,52] However, before absorption inhibition mechanisms are considered, the amount of micellised β-carotene applied to the Caco-2 cells is a major factor affecting β-carotene absorption. The hypothesis in this study is that the decreased micellization of β-carotene is, to some extent, due to the reduction of micelle formation. This would also mean that there is less substrate (e.g. free fatty acid, bile salts) which facilitates the transport of β-carotene through the enterocyte. Both plasma carotenoid response in humans and carotenoid uptake by Caco-2 cells are linearly related to the carotenoid dose, when ingested at nutritional doses or applied at physiological concentrations, respectively.[49] In this study the amount of β-carotene taken up by the Caco-2 cells was strongly positively correlated ($R^2 = 0.74$, $p<0.001$) with the amount of β-carotene applied to the Caco-2 cells. This suggested that little additional specific inhibition of β-carotene uptake by the Caco-2 cells occurred.

An additional experiment was performed to determine if the reductions in β-carotene uptake, when digested with fibre, iron or phenolics, were due to specific inhibition of uptake by the Caco-2 cells or just because lower amounts of β-carotene were added to the Caco-2 cells (Figure 5B). β-carotene was digested alone and filtered (0.2 μM) to obtain the mixed micellar fraction (Figure 1).

After this, pectin, iron, ferulic acid, catechin and naringenin were separately added to the micellized β-carotene just before the 4 h-incubation with the Caco-2 cells. Under these conditions iron, ferulic acid and catechin did not significantly reduce all-trans β-carotene uptake (**Figure 5B**). The concentrations of iron, ferulic acid and catechin added to

the micellar fraction before the Caco-2 cell incubation was then doubled and still, there were no reductions in all-trans β-carotene uptake (results not shown).

Naringenin and the phenolic mix, however, significantly reduced all-trans β-carotene uptake (**Figure 5B**). It has been found that naringenin inhibited transepithelial transport of lutein through Caco-2 cells,[25] and inhibition of scavenger receptor class B type I (SR-BI), a membrane transporter involved in carotenoid uptake, was given as the mechanism.[53] Recently, however, Nie et al.[26] also found that naringenin decreased the β-carotene uptake into Caco-2 cells, but interestingly, that naringenin increased the expression of SR-BI, indicating a complex effect of naringenin on β-carotene absorption.

In conclusion, at molar ratios similar to those in green leafy vegetables, neither pectin, iron, ferulic acid, nor catechin inhibited β-carotene uptake by Caco-2 cells. Our findings support the notion that these compounds interfere with digestive processes (enzyme inhibition and bile salt precipitation) in the small-intestinal lumen rather than the absorption of β-carotene.

The model system used in this study provided the opportunity to identify the effect of individual matrix factors on β-carotene bioaccessibility, which is impossible when the whole food is analysed. It should, however, be kept in mind that, in this model system, compounds can easily interact as it does not include the effects of the whole food matrix. Nevertheless, other authors have compared model systems evaluating the effect of phenolics on β-carotene micellization to whole food systems with similar results.[45] It is also important to keep in mind, when interpreting the β-carotene solubility and micellization results, that the *in vitro* digestive assays are static and digestive juices are limited and not constantly replenished, as would be the case in the human body. In vitro digestive systems

used also vary, including different digestive stages including oral and stomach digestion not used in this study and also using different amounts of enzymes and digestive additive such as bile extracts. Not all possible mechanisms of micellization inhibition were analysed in this study, such as free fatty acid precipitation and micellar characteristic, which would have shed light on additional mechanisms. It should also be noted that in this study, higher concentrations of the test compounds were included in the digestion (equal to 0.6 g leafy vegetable/ml) as would be from normal consumption of green leafy vegetables.

This is the first study showing the inhibitory effect of individual matrix factors (iron, phenolics) of green leafy vegetables on aspects of β-carotene bioavailability. This information can be used to optimise green leafy vegetable processing (e.g. soaking to remove contamination iron, which has been found to be not bioaccessible[54]) and meal formulations (e.g. consumption of fruits containing organic acids which can chelate iron) to increase the β-carotene bioavailability from these meals. It should also be noted that while this study only evaluated the effect of iron, green leafy vegetables are also rich in other divalent minerals such as calcium, iron and zinc,[12] which have also been shown to reduce carotenoid micellization,[19] and could possibly impact β-carotene bioavailability even further. The degree to which the reduced micellization is physiologically relevant, where bile salts and pancreatic lipase are constantly secreted during intestinal digestion, should also be determined in *in vivo* models or, preferably, human trials.

Abbreviations: Only standard abbreviations used

Supporting information description: Data on the bioaccessibility and uptake of 9-cis- and 13-cis-β-carotene are available as supplementary data (**Supplementary Figures S2-S10**).

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6. Conflict of Interest:

The authors declare that they have no known conflict of interest.

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