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Negative impact of soluble, gel-forming dietary fibres on the bioaccessibility of β -carotene, lutein, and lycopene†

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Carotenoids, including β -carotene, lutein and lycopene, the 3 main carotenoids present in our body, are lipophilic phytochemicals associated with several health benefits. Dietary fibre may influence the bioavailability of carotenoids by altering their release from the food matrix and their solubilisation into mixed micelles during digestion and thus their subsequent absorption by enterocytes. We examined the dose-dependent influence of major soluble (pectin, guar, alginate, fructooligosaccharides,) and insoluble dietary fibres (cellulose, type-2 resistant starch) at nutritional relevant doses (0, 30 and 90 mg per 26 ml) added for digestion, containing also pure β -carotene, lutein or lycopene (at 75 μ g) solubilized in peanut oil. Following *in vitro* gastrointestinal digestions, carotenoid bioaccessibility, selected physico-chemical parameters (viscosity, surface tension, ζ -potential and micelle size) and free fatty acid release were evaluated. β -Carotene bioaccessibility was reduced by 90 mg of alginate and pectin, from 29.1% to 11.8% and 17.9%, respectively ($p < 0.001$), while other fibres had no overall significant impact. For lutein, only pectin decreased its bioaccessibility, from 58.3% to 26.0% ($p < 0.001$), while for lycopene, the reduction in bioaccessibility was from 7.2% (control) to 5.4% for pectin ($p < 0.05$), 4.1% for alginate ($p = 0.001$) and 4.8% for guar ($p < 0.05$) for 90 mg. This negative effect was associated with altered physico-chemical properties, with soluble, gel-forming fibres (vs. non-gel-forming fibres) in general increasing viscosity, reducing surface tension and absolute ζ -potential and in part micelle size, and hampering triglyceride digestion by up to 54.3% for guar (90 mg) vs. controls. Thus, soluble, gel-forming dietary fibres hampered carotenoid bioaccessibility, also depending on carotenoid type, while insoluble and non-gel-forming dietary fibres showed no negative effect on bioaccessibility.

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Introduction

Carotenoids constitute a range of phytochemicals synthesized by photosynthetic organisms such as algae, plants, and bacteria, as well as by certain non-photosynthetic fungi, archaea, protists, bacteria¹ and certain arthropods.² Although non-essential for humans, there is epidemiological evidence³ that the consumption of several carotenoids is associated with a number of health benefits, such as a reduced risk of cardiovascular diseases (CVDs),⁴ oxidative stress⁵ cancer, age-related

macular degeneration (AMD)^{6,7} and inflammation.⁸ However, carotenoid bioactivity relies on their bioavailability, *i.e.* the fraction of a nutrient that is absorbed and can be used for its intended physiological action,⁹ which is typically low and variable, often below 30%.¹⁰ Carotenoids are extremely lipophilic and are absorbed through pathways akin to those of dietary lipids. Their bioavailability is a function of a multitude of intrinsic factors such as gut microbiota composition,¹¹ amount of adipose tissue and inflammatory state¹² age, sex,¹³ gender,¹⁴ genetic makeup,^{15,16} hormonal fluctuations,¹⁷ food processing such as heat treatment,¹⁸ food matrix related factors such as presence of proteins, dietary lipids, and divalent minerals^{19,20} and finally lifestyle related ones such as medication.²¹

A factor that remains understudied with respect to dietary factors influencing the bioavailability of carotenoids is dietary fibre (DF), often defined as non-digestible carbohydrates plus lignin.²² DF may exert considerable influence on the bioaccessibility (defined as the fraction of an ingested compound that is biochemically and/or physically released from the food matrix

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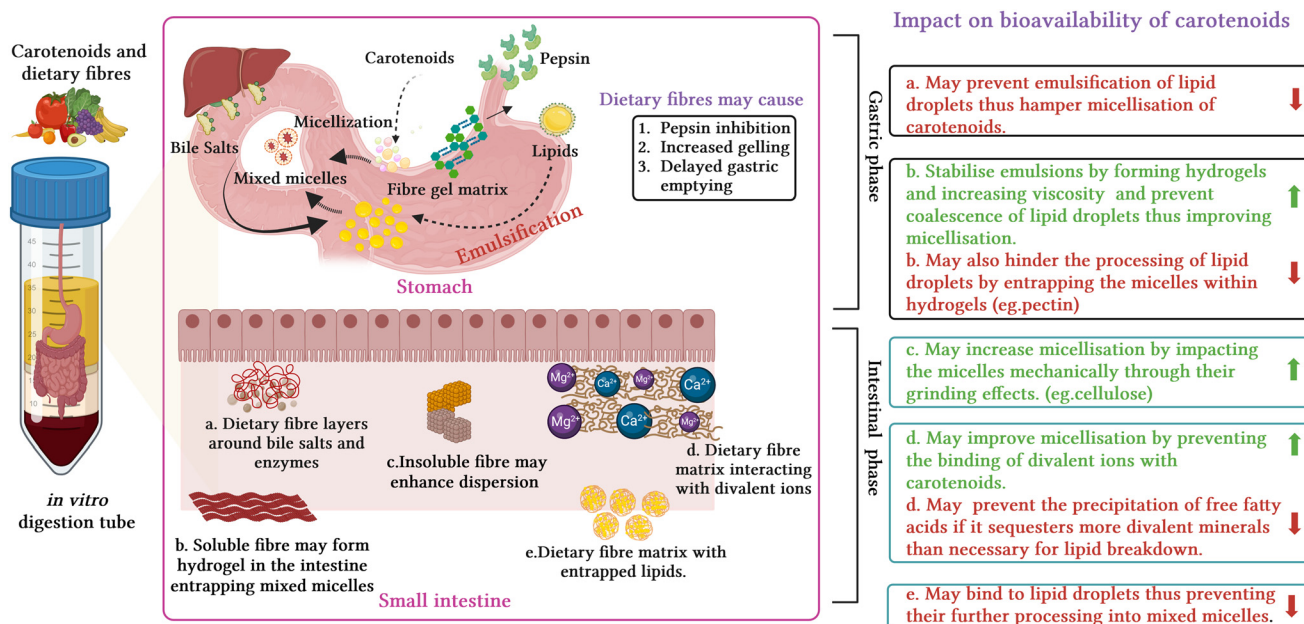


Fig. 1 Potential effect of dietary fibres on bioavailability of carotenoids.

and solubilized within the gastrointestinal fluids in an absorbable form²³ of fat-soluble compounds, including carotenoids, by modifying structural and physical properties of the digesta, contingent upon their type, molecular composition such as molecular weight, and physiological characteristics such as water solubility or gelling properties.^{24,25}

DF may alter viscosity,^{26–28} stabilize emulsions,^{29,30} form gel matrices that entrap lipids,²⁸ bind to organic molecules and ions such as Ca^{2+} ,³¹ all factors that could modulate the bioaccessibility of carotenoids (Fig. 1). DF can be present in the digestive tract in 3 main physical forms: as soluble polymer chains in solution, as insoluble macromolecular assemblies, and as swollen, hydrated, sponge-like networks.³² The principal physiological effect of DF in the small intestine is to reduce the rate (and in some cases the extent) of release of a number of nutrients or phytochemicals.³³ The key factors regarding the impact of DF on the digestion of other food constituents include (i) their physical entrapment within structured assemblies;³⁴ (ii) enhanced viscosity of gastric/intestinal fluids restricting the peristaltic mixing process that promotes transport of enzymes to their substrates, bile salts to unmicellized fat, and nutrients to the gut wall;³⁵ and (iii) enzymes or bile salts essential for the solubilisation of food constituents may also bind to DF.

Certain DF, such as pectin, can enhance gelling properties, *i.e.* forming a cross-linked hydrophilic polymer network, which may slow down the release of carotenoids from the food matrix.³⁶ In one study, supplementing a controlled meal with citrus pectin significantly reduced the concentration of β -carotene (supplied *via* a capsule) in plasma.³⁷ In another study, female participants were supplemented with an antioxidant mixture with or without DF (pectin, guar, alginate, cellulose, or wheat bran) added to a standard meal. The 24 h

area under curve of the plasma carotenoid concentration showed that water-soluble and gel-forming fibres (pectin, guar, alginate) significantly reduced the area under curve for β -carotene lutein and lycopene by 33–43%.³⁸ Inulin, also a soluble gel-promoting fibre,³⁹ added to tomato sauce, decreased the bioaccessibility of lycopene (by 56–62%), β -carotene (by 32–59%), and lutein (28–56%).⁴⁰ Some DF can bind to divalent ions (such as Ca^{2+} and Mg^{2+}), depending on the type of DF, pH and ionic strength, *via* carboxyl and hydroxyl groups.⁴¹ Such interactions could even exert positive effects on carotenoid bioavailability, as earlier studies have demonstrated that Ca^{2+} and Mg^{2+} , at high but still physiological doses, could hamper bioaccessibility and bioavailability of carotenoids.^{42,43}

The current study aimed at a more systematic investigation of the effect of various frequently consumed and different types of DF on carotenoid bioaccessibility and selected physico-chemical properties of the digesta, including lipid digestion, by means of the INFOGEST European consensus model for static *in vitro* digestion.⁴⁴ Pectin, guar, alginate, cellulose, resistant starch type-2 (RS II) and fructooligosaccharides (FOS) were co-digested individually with β -carotene, lutein and lycopene (Tables 1 and 2).

Materials and methods

Carotenoid standards, chemicals and enzymes

Unless otherwise stated, all chemicals were of analytical grade or superior. Analytical standards of all *trans* form of β -carotene (purity $\geq 95\%$) (Art. No. PHR1239) were obtained from Sigma-Aldrich (Overijse, Belgium). Lutein (purity $\geq 92\%$) (Art. No.



Table 1 Overview of the applied conditions and parameters investigated following the simulated gastrointestinal (GI) digestion

Digestion steps	Dietary fibre type	Carotenoid type	Micellar phase carotenoids	Lipid digestion aspects	Physicochemical characteristics	
					Solution	Particles
Gastric and intestinal phases	<ul style="list-style-type: none"> • Pectin • Alginate • Fructooligosaccharides • Resistant starch type II • Guar • Cellulose 	<ul style="list-style-type: none"> • β-Carotene • Lutein • Lycopene 	Bioaccessibility	Free fatty acid release	<ul style="list-style-type: none"> • Macro-viscosity • Surface tension 	<ul style="list-style-type: none"> • ζ-Potential • Micelle size

Table 2 Different properties of the selected dietary fibres investigated in the study

DF type	Natural sources and concentration in food	Structural basis (main units)	Molecular weight (Da)	Solubility in water	Fermentability
Pectin	Apple peel (14.5% of dw ^a) Apple pomace (33.5% of dw) ⁴⁸	Galacturonic acid with methoxy groups ⁴⁵	50 000 to 300 000 ^{46,47}	Yes ⁴⁵	Yes ⁴⁵
Alginate	Brown algae ⁴⁹	β -(1,4)-D-Mannuronic acid, α -(1,4)-L-Guluronic acid ⁴⁵	60 000 to 710 000 ⁵⁰	Yes ⁴⁵	Yes ⁴⁵
Resistant starch type 2	Sweet potato (54.9 g per 100 g) Bread crumbs (62.3 g per 100 g) ⁵²	Amylose, amylopectin ⁴⁵	31 090 000 to 48 200 000 ⁵¹	No or poorly ⁴⁵	Not readily ⁴⁵
Guar	Guar plant Tomato ketchup (1%) ^{45,53}	β -(1,4)-D-Mannose ⁴⁵	50 000 to 8 000 000 ⁵⁰	Yes ⁴⁵	Yes ⁴⁵
Cellulose	All plants, some algae and bacteria ⁴⁵	β -(1,4)-D-Glucose ⁴⁵	60 000 to 180 000 ⁵⁴	No ⁴⁵	Low ⁴⁵
Fructooligosaccharides	Chicory root (19.6 g per 100 g) Bran-raw wheat (1 g per 100 g) Raw leeks (2.4 g per 100 g) ⁵⁶	D-Fructose ⁴⁵	Up to 3500 ⁵⁵	Yes ⁴⁵	Yes ⁴⁵

^a Acronym: dw = dry weight.

0306S) and all-*trans* lycopene (purity \geq 95%) (Art. No. 305S) were both obtained from Extrasynthese (Genay, France). Pepsin from porcine gastric mucosa (\geq 250 U mg⁻¹, Art. No. P7000), pancreatin from porcine pancreas (activity equivalent to 4 \times USP specifications, Art. No. P1750) and porcine bile extract (Art. No. B8631) were obtained from Sigma-Aldrich. Potassium chloride (\geq 99%), potassium dihydrogen phosphate (\geq 99%), sodium hydrogen carbonate (\geq 99%), sodium chloride (\geq 99.5%), magnesium chloride hexahydrate, ammonium carbonate, sodium hydroxide solution (1 M), and calcium chloride dihydrate (\geq 99%) were from Sigma-Aldrich. *n*-Hexane (\geq 95%), acetone (\geq 99%), and hydrochloric acid (1 M) were from VWR (Leuven, Belgium).

Dietary fibres and lipid sources

Dietary fibres were chosen based on their frequency of consumption and differing characteristics, *i.e.* being water soluble *vs.* non-soluble.⁵⁷ Cellulose (Art. No. 22183), fructooligosaccharides (FOS) from chicory (Art. No. F8052), guar powder from guar plant (Art. No. G4129), alginic acid sodium salt (Art. No. 180947, termed alginate in the remainder of the manuscript), and pectin from apple (Art. No. 93854, degree of esterification 50–75%) were obtained from Sigma-Aldrich, while resistant starch II (RS II) (granular, with high crystalline structure) derived from pulverized green bananas (60.0% pure DF) was obtained from Jonny's Good Nature LLC (Ohio, USA).

Peanut oil, free of innate carotenoids based on literature⁵⁸ and own examinations, was purchased from a local supermarket (Delhaize, Strassen, Luxembourg).

Solubilisation of carotenoid standards & dietary fibres

All solutions were prepared using the protocol previously described.⁵⁹ The concentration for each of the carotenoid standards in oil was 0.5 mg mL⁻¹ for a delivery of 75 μ g carotenoids (in 150 μ l oil) per digestion, reflecting a high but achievable daily intake of 28 mg carotenoids per assumed 10 L of daily secreted digestive fluids⁶⁰ or 5–6 mg per 2 L of digestive fluid simulating rather a single meal.⁶¹ β -Carotene and lycopene standards were initially solubilized in hexane, followed by subsequent addition of peanut oil to produce a solution of carotenoids in oil, while lutein was solubilized in acetone first and then in peanut oil. Peanut oil was selected as the lipid vehicle due to its minimal intrinsic carotenoid content, good oxidative stability, and its proven capacity to dissolve and stabilize carotenoids effectively in *in vitro* digestion models, as confirmed by both literature and our own analysis.⁵⁸ The final solution in oil was sonicated for 5 min at 25 °C at 50–60 Hz using Ultrasonic Cleaner (VWR Symphony®, MA), and kept in a water bath pre-heated at 40 °C for 5 min prior to use. The organic solvents were evaporated under a stream of nitrogen (TurboVap LV from Biotage®, Uppsala, Sweden) and the carotenoid parental solution in oils were made by complete evaporation of organic solvents and further addition of peanut oil to



the carotenoid solution to reach the targeted concentration of 0.5 mg mL^{-1} .

The recommended adequate intake (AI) for DF as recommended by the European Food Safety Authority (EFSA) and the Food and Agriculture Organization (FAO), is 25 g per day per adult.²² When downscaled to the *in vitro* investigation, this translated into approximately 65 mg of DF per digested sample, considering a total digestive volume of 10.0 L per day.⁶⁰ In order to cover a range of DF consumption, the three doses investigated were 0 mg, 30 mg and 90 mg per 26 mL of digesta. These concentrations corresponded to approx. 0, 46, and 138% of the AI for DF.

Simulated gastric fluid and simulated intestinal fluid

The simulated gastric fluid (SGF), the simulated intestinal fluid (SIF), as well as the enzyme solutions were prepared as recommended by INFOGEST2.0.⁴⁴ Pepsin solution was prepared in SGF at a concentration of 2000 U mL^{-1} of the 13.0 mL gastric mixture, pancreatin and bile extract were prepared in the same SIF solution, at a concentration of 200 U mL^{-1} (the amount of pancreatin added is based on the trypsin activity 100 U mL^{-1} in the 26.0 mL mixture)⁴⁴ and 6.8 mg mL^{-1} , respectively. Given that the food matrix was liquid and lacked carbohydrates, the oral phase was excluded from the *in vitro* digestion model.

Gastric phase

Each sample comprised a total of 6.5 mL of gastric phase solution containing the desired dose of DF *i.e.* 0, 30 or 90 mg, as well as 150 μL of peanut oil containing the respective carotenoid standards (approx. 75 μg of carotenoid standard per digestion). Prior to the addition of SGF ($1.25 \times$ concentrate), 2000 U mL^{-1} of pepsin and calcium chloride to reach a final concentration of 0.075 mM were added to the samples. Before incubation, pH was adjusted to 3.0 by hydrochloric acid (1.0 M), bringing the volume of each sample to 13.0 mL with pure water in order to reach a final ratio of the matrix (DF and carotenoid solutions) to simulated gastric fluid of 50/50 (v/v). Samples were then incubated in a shaking water bath (GFL 1083 from VEL®, Leuven, Belgium) for 2 h at $37 \text{ }^\circ\text{C}$, with a shaking speed of 100 rotations per minute (rpm).

Intestinal phase

At the end of the gastric incubation, SIF, pancreatin (200 U mL^{-1}) and bile extract (6.8 mg mL^{-1}) were added to the chyme. Calcium chloride was added individually at a concentration of 0.3 mM in the final mixture. The ratio of gastric chyme to SIF of 50/50 (v/v) was obtained by filling up the samples up to 26 mL with pure water, the pH was adjusted to 7 by the addition of sodium hydroxide solution (1 M). The tubes were then sealed with parafilm before incubating them for 2 h at $37 \text{ }^\circ\text{C}$, maintaining a shaking speed of 100 rpm.

Analyses of digested samples

Extraction and analysis of the bioaccessible phase.

Carotenoid extraction and analyses were carried out as described by Iddir *et al.*⁵⁹ Briefly, at the end of the intestinal

incubation, the samples were centrifuged at 3400g for 1 h at $4 \text{ }^\circ\text{C}$. Subsequently, 9.0 mL of the middle aqueous phase (to avoid any potential remaining lipid droplets from being collected) from each sample was carefully collected and passed through a $0.2 \mu\text{m}$ nylon membrane syringe filter (Whatman Puradisc 25, Art. No. 6751-2502). The extraction started by adding 6.0 mL of *n*-hexane: acetone (2 : 1, v/v) to 3.0 mL of the filtered aqueous phase for β -carotene and lycopene while 2.0 mL were used for lutein (to account for the typical higher bioaccessibility), followed by thorough vortexing. After a brief centrifugation, the organic phase was collected, and the extraction process was repeated two times with pure *n*-hexane. Organic phases were pooled in the same tube, dried under a stream of nitrogen, and stored under argon at $-80 \text{ }^\circ\text{C}$ until the spectrophotometric measurements.

For spectrophotometric analyses, 750 μL of *n*-heptane (VWR BDH Chemicals) was added to the recovered carotenoid pellet and the absorbance was monitored between 300 and 700 nm (GENESYS™ 10S UV-Vis spectrophotometer, ThermoFisher Scientific, Waltham, MA) as described previously (Iddir *et al.*, 2019);⁵⁹ Beer–Lambert's law was used to calculate the amount of carotenoids extracted from the digesta, where the absorbance was measured at 450 nm and 470 nm for β -carotene and lycopene, respectively⁶² and 445 nm for lutein.⁶ A molar absorption coefficient for lycopene and β -carotene in *n*-heptane of $138\,824 \text{ L (mol cm)}^{-1}$ and of $139\,000 \text{ L (mol cm)}^{-1}$ for lutein were used.⁶³ Digestive blanks were run to assure the absence of other absorbing components at 445, 450 and 470 nm.

The percentage of carotenoid micellization was then employed as a measure of bioaccessibility, and was expressed as the percentage of amount of carotenoids present in the micellar phase of the filtered digesta after *in vitro* GI digestion, compared to the initial amount added to the sample.

Physico-chemical characterization of digesta

Viscosity. Viscosity was assessed as earlier studies found associations with carotenoid bioaccessibility.⁵⁹ The viscosity of the unfiltered digesta was investigated using a double gap cylinder configuration in a MCR 302 WESP rheometer from Anton Paar (Graz, Austria). In brief, the frozen samples were thawed shortly before the measurement. Three samples of each type were measured as follows: after temperature equilibration to $5 \text{ }^\circ\text{C}$, a pre-shear phase with a shear rate of 5 s^{-1} was applied for 30 s. Then, the viscosity as a function of shear rate was determined between 0.1 and 131 s^{-1} (78 measurement points), first in an increasing and then in a decreasing mode. Additionally, a stabilisation period of about 30 s was applied between the rising and falling mode with a shear rate of 131 s^{-1} . Increasing and decreasing data were merged because hysteresis were not observed. The curves of the sample type were averaged and evaluated in the shear rate range from 1 to 131 s^{-1} .

Surface tension

Surface tension reduction has been related to the presence of surface active compounds, which may promote emulsion



stability,⁶⁴ and for this reason were measured in this study. Surface tension of digesta samples, pre-conditioned at 20.0 ± 0.1 °C, were determined by the weight-drop method and a self-assembled apparatus adopted by.⁶⁵ The air-water interfacial properties of the digesta were calculated as follows:

$$\sigma_{\text{digesta}} = \frac{M_{\text{digesta}}}{M_{\text{H}_2\text{O}}} \times \sigma_{\text{H}_2\text{O}}$$

where $\sigma_{\text{H}_2\text{O}} = 72.5$ mN m⁻¹ at 20 °C,⁶⁵ $\sigma_{\text{H}_2\text{O}}$ being the surface tension of pure water, σ_{digesta} being the surface tension of digesta, M_{digesta} being the mass of one drop of digesta and $M_{\text{H}_2\text{O}}$ being the mass of one drop of water and mN be milli Newton and m is meters.

Micelle size and ζ -potential analysis

Both micelle size⁶⁶ and ζ -potential⁶⁷ may impact the stability of an emulsion and for that reason were included in the measurements. Aliquots from the micellar phase were used for the analysis of the micelle size and ζ -potential, and the measurements were done at 25 °C temperature with at least four independent digesta replicates. The intensity-weighted mean hydrodynamic radius and ζ -potential were determined by dynamic light scattering and laser Doppler micro-electrophoresis, respectively, by using a Zetasizer Nano Zs instrument (Malvern Instruments, Malvern, UK).

Free fatty acid analysis

Diacylglycerols formed, as well as free fatty acids (FFA) can increase emulsification and mixed micelle formation⁶⁸ and were thus assessed. Quantification of free fatty acids was done following the gastrointestinal digestion to estimate the extent of triglyceride (TG) lipolysis. This was accomplished by employing the Cayman Free Fatty Acid Fluorometric Assay kit and the protocol provided by the manufacturer (Cayman Chemical, Art. No. 700310, Ann Arbor, MI).

Sample preparation

Aliquots of filtered digesta were stored at -80 °C, then thawed and brought to room temperature prior to analysis. Samples were diluted 1 : 40 using the kit's FFA Sample Buffer.

This dilution factor was selected based on the expected oleic acid concentration, estimated through the calibration curve provided by the supplier, to ensure values fell within the assay's dynamic range and to allow for the detection of up to 100% theoretical lipolysis.

Statistical analysis and data treatment

In order to minimize day-to-day variations between experiments, bioaccessibility of pure carotenoids was normalized to a daily control as described earlier,⁶⁹ which was assessed for each digestion. Unless otherwise stated, all values are expressed as the mean \pm SD. Statistical analysis was performed using SPSS 25 software (SPSS Inc., Chicago, IL). Normality plots were created to confirm the normal distribution of data, together with box plots to assess equality of variance. Linear

mixed models were performed with carotenoid bioaccessibility as the dependent, observed outcome, while type ($n = 6$) and concentration (3 quantitative levels) of DF and type of carotenoid were fixed independent factors. Upon identifying significant interactions, supplementary linear mixed models were employed, controlling for specific parameters to enable more individual group-wise comparisons. Non-significant interactions were removed one by one from the models. Statistical significance was defined by a two-tailed p -value < 0.05 . Where appropriate, *post-hoc* analyses with Tukey's test were applied for comparisons involving more than three groups, and the Fisher-protected least significant difference (LSD) test for comparisons of three or fewer groups. For studying correlations between various outcomes, Spearman rank correlations were calculated.

Results

General influence of DF on the bioaccessibility of carotenoids

Overall, *i.e.* considering all pooled results (*i.e.* including all doses and types of DF), the bioaccessibility of lutein (as specified by the estimated marginal means of the model) was $57.0 \pm 8.5\%$ (Fig. 2A), followed by β -carotene $26.8 \pm 6.3\%$ (Fig. 2B) and lycopene $6.7 \pm 1.2\%$, (Fig. 2C) all of which were significantly different from each other ($p < 0.05$).

Overall, DF and dosage had a significant impact on carotenoid bioaccessibility, as indicated by the general linear mixed model ($p < 0.001$ for main effect). Significant interactions were observed, including DF \times carotenoid types, DF dose \times carotenoid type, DF type \times dose, as well as carotenoid type \times DF dose ($p < 0.001$). These results suggest that the effects varied depending on the specific type of DF and its dose used. This was confirmed by the overall general estimated marginal means, showing lowest bioaccessibility for pectin $25.7 \pm 18.7\%$ and alginate $28.2 \pm 23.2\%$ followed by guar at $30.6 \pm 21.5\%$, compared to controls ($p < 0.05$). Contrarily, the impact of non-gel forming fibres cellulose, FOS and RS II were minimal and not significantly different from controls, *i.e.* $32.1 \pm 22.1\%$, $32.2 \pm 21.8\%$ and $32.1 \pm 22.3\%$, respectively, considering all carotenoids and DF doses were pooled (Fig. 3).

For β -carotene, alginate significantly lowered the bioaccessibility from $29.1 \pm 4.5\%$ at 0 mg (controls) to $17.7 \pm 3.9\%$ at 30 mg ($p = 0.010$) and further to $11.8 \pm 3.7\%$ ($p = 0.001$) at 90 mg, respectively, while pectin only had a significant effect at 90 mg, *i.e.* $17.9 \pm 2.4\%$ ($p = 0.001$), when compared to the DF-free control. Interestingly, the mean bioaccessibility for the samples without DF (controls) was comparable to those with FOS, guar, RS II and cellulose ($p > 0.05$).

Similarly, for lutein, pectin had a significant negative impact on its bioaccessibility $26.0 \pm 3.0\%$ at 90 mg compared to controls at $58.3 \pm 1.9\%$ ($p < 0.001$). Another significant impact was observed for RS II, where the impact on bioaccessibility for both the DF doses was small but significant ($p = 0.017$) at 30 and 90 mg, being the same at $61.9 \pm 1.0\%$, compared to controls $58.3 \pm 1.9\%$. Apart from pectin and RS II, all



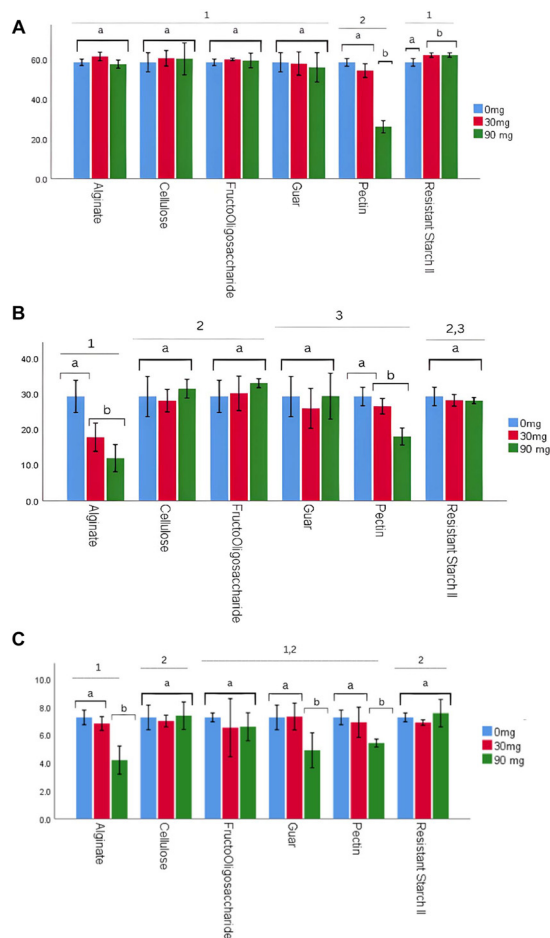


Fig. 2 (A) Mean bioaccessibility (%) of lutein in the presence of different dietary fibres – alginate, cellulose, FOS (fructooligosaccharides), guar, pectin, and resistant starch II (RS II). Three different fibre quantities compared were 0 mg (blue), 30 mg (red), and 90 mg (green). Data are expressed as mean bioaccessibility percentage \pm standard deviation. Error bars represent standard deviation across three quantities 0 mg, 30 mg, and 90 mg. The mean bioaccessibility for lutein with all concentrations and fibre types pooled was $57.0 \pm 8.5\%$. All group-wise comparisons were carried out by Tukey's *post hoc* test, with fibres categorized into subsets 1 and 2. Within each fibre type, different letters (a and b) indicate significant differences among the three doses. (B) Mean bioaccessibility (%) of β -carotene in the presence of different dietary fibres – alginate, cellulose, fructooligosaccharides (FOS), guar, pectin, and resistant starch II (RS II). Three different fibre quantities compared were 0 mg (blue), 30 mg (red), and 90 mg (green). Data are expressed as mean bioaccessibility percentage \pm standard deviation. The mean bioaccessibility for β -carotene with all concentrations and fibre types pooled was $26.8 \pm 6.3\%$. All group-wise comparisons were done using Tukey's *post hoc* test, with fibres categorized into subsets 1, 2. Within each fibre type, different letters (a and b) indicate significant differences among the three doses. (C) Mean bioaccessibility (%) of lycopene in the presence of different dietary fibres – alginate, cellulose, FOS (fructooligosaccharides), guar, pectin, and resistant starch II (RS II). Three different fibre quantities compared were 0 mg (blue), 30 mg (red), and 90 mg (green). Data are expressed as mean bioaccessibility percentage \pm standard deviation. Error bars represent standard deviation across three quantities 0 mg, 30 mg, and 90 mg. The mean bioaccessibility for lycopene with all concentrations and fibre types pooled was $6.7 \pm 1.2\%$. All group-wise comparisons were carried out by Tukey's *post hoc* test, with fibres categorized into subsets 1 and 2. Within each fibre type, different letters (a and b) indicate significant differences among the three doses.

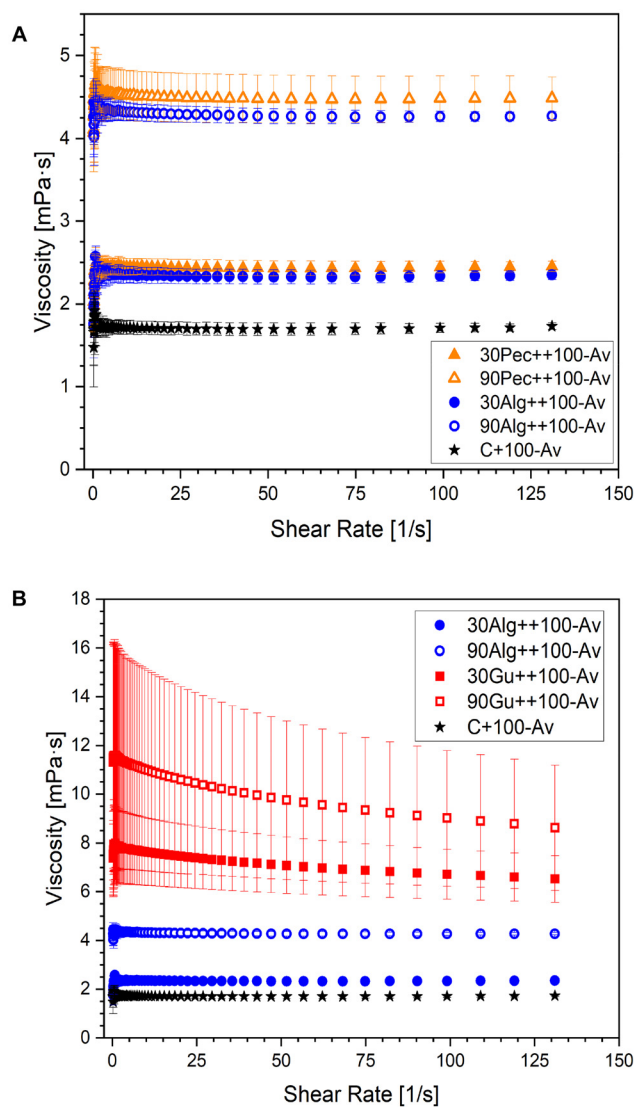


Fig. 3 (A) Viscosity vs. shear rate plot comparing the viscosity of pectin and alginate at 30 and 90 mg. The x-axis represents the shear rate [1/s], while the y-axis shows viscosity [mPa s], for pectin at 30 mg (closed orange triangles) and 90 mg (open orange triangles) and alginate at 30 mg (closed blue circles) and 90 mg (open blue circles) and the control (black stars). (B) Viscosity vs. shear rate plot comparing the viscosity of guar and alginate at 30 and 90 mg. The x-axis represents the shear rate [1/s], while the y-axis shows viscosity [mPa s], for guar at 30 mg (closed red squares) and 90 mg (open red squares) and alginate at 30 mg (closed blue circles) and 90 mg (open blue circles) and the control (black stars).

other DF conditions (FOS, cellulose, guar and alginate) did not have a statistically significant effect on lutein compared to controls, considering all doses.

The bioaccessibility of lycopene was significantly negatively impacted by alginate reducing it to $4.1 \pm 1.0\%$ ($p < 0.001$), guar to $6.4 \pm 1.2\%$ ($p = 0.01$) and pectin to $5.4 \pm 0.2\%$ ($p = 0.01$) at 90 mg compared to controls $7.2 \pm 0.5\%$. Cellulose, RS II and FOS had no significant impact.



Influence of individual dietary fibre on the bioaccessibility of carotenoids

Alginate. Overall, *i.e.* considering various types of carotenoids and dosing, alginate significantly influenced carotenoid bioaccessibility ($p < 0.001$), with a significant interaction term between carotenoid type and dosing ($p < 0.001$).

The addition of alginate to the digested samples had a significant impact on the bioaccessibility of β -carotene and lycopene at both concentrations (30 and 90 mg). The decline in bioaccessibility upon addition of alginate from 0 to 30 mg was from $29.1 \pm 4.5\%$ to $17.7 \pm 3.9\%$ and further to $11.8 \pm 3.7\%$ at 90 mg for β -carotene and from $7.2 \pm 0.5\%$ to $6.8 \pm 0.5\%$ at 30 mg and $4.1 \pm 1.0\%$ at 90 mg in case of lycopene. The increase of 30 vs. 90 mg of alginate impact lutein bioaccessibility significantly.

When comparing alginate to other DFs, the impact of alginate on carotenoid bioaccessibility was significantly different from all the other DF groups in case of β -carotene ($p < 0.05$) (including control group), while it was comparable to other DF groups in case of lutein except for pectin ($p < 0.001$). Moreover, in case of lycopene, the behaviour of alginate was significantly different only from cellulose and RS II, ($p < 0.05$) and showed behaviour comparable to pectin, guar and FOS.

Cellulose

Similar to FOS, neither cellulose dosage independently nor the interaction between DF dosing and carotenoid type had no significant impact on carotenoid bioaccessibility. However, the impact of cellulose was significantly dependent on the type of carotenoid ($p < 0.001$). The impact of cellulose on carotenoid bioaccessibility was significantly different from pectin ($p = 0.043$) and alginate ($p < 0.001$) in case of β -carotene, and only different from pectin in case of lutein ($p < 0.001$), and marginally different from alginate in case of lycopene ($p = 0.056$).

Fructooligosaccharides

FOS dose did not significantly influence carotenoid bioaccessibility considering the dosage, although the impact was significant for each carotenoid type ($p < 0.001$); the interaction term of FOS dose and carotenoid type was not significant.

For β -carotene, the effect of FOS was significantly different from alginate ($p < 0.001$), and pectin ($p = 0.040$), while being in the same range as cellulose, RS II, guar and control. For lutein, the impact of FOS was significantly different only from pectin ($p < 0.001$), while for lycopene, it was not significantly different from other DF.

Guar

Overall, *i.e.* considering all various types of carotenoids and dosing, guar did not significantly influence carotenoid bioaccessibility. With respect to individual carotenoids, guar did not show a significant effect on the bioaccessibility of β -carotene and lutein compared to the control group, however it did have a negative impact on the bioaccessibility of lycopene, negatively impacting it from $7.2 \pm 0.8\%$ for controls to $4.8 \pm 1.2\%$ at 90 mg ($p = 0.033$).

For β -carotene bioaccessibility, the effect of guar was found to be significantly different from alginate ($p < 0.001$), but not from other DF or controls. For lutein, the impact of guar was not distinct compared to all the other DF and control groups but differed significantly from pectin ($p < 0.001$). However, in case of lycopene, guar showed no significant difference vs. other DF groups.

Pectin

Overall, *i.e.* considering various types of carotenoids and dosage pooled, pectin significantly reduced carotenoid bioaccessibility ($p < 0.001$) in addition to exhibiting a significant interaction between carotenoid type and dosing ($p < 0.001$).

Pectin showed a significant impact on the bioaccessibility of all the three carotenoids at 90 mg ($p < 0.05$), reducing it from $29.1 \pm 2.6\%$ to $17.9 \pm 2.3\%$ for β -carotene, while from $58.3 \pm 1.9\%$ to $26.0\% \pm 3.0\%$ for lutein and from $7.2 \pm 0.5\%$ to $5.4 \pm 0.2\%$ for lycopene. The impact of pectin at 30 mg was not significant on all species of carotenoids.

A comparison with other DF groups revealed that the impact of pectin on carotenoid bioaccessibility was significantly different from alginate ($p = 0.049$), cellulose ($p = 0.043$) and FOS ($p = 0.004$), although not from guar and RS II in case of β -carotene. For lutein, pectin was significantly different from all the other DF groups and controls ($p < 0.001$). In case of lycopene, pectin did not show a difference of behaviour compared to other DF types.

Resistant starch type-II

RS II had no significant impact on overall carotenoid bioaccessibility; however, the impact differed significantly ($p < 0.001$) depending on carotenoid type. Subsequently, the interaction between RS II dose and carotenoid type were found to be significant ($p = 0.007$). The effect of RS II on carotenoid bioaccessibility however differed significantly from that of alginate ($p < 0.001$) in case of β -carotene; differed from pectin ($p < 0.001$) in case of lutein and differed from alginate ($p = 0.040$) in case of lycopene. An increase in dosage of RS II exhibited no significant impact on the bioaccessibility of β -carotene and lycopene but a significant impact on the bioaccessibility of lutein ($p = 0.017$) upon moving from 0 to 30 mg, altering the bioaccessibility from $58.3 \pm 1.9\%$ to $61.9 \pm 1.0\%$. However, no significant difference was observed upon moving from 30 to 90 mg.

Effect of dietary fibres on different physico-chemical properties of digesta

Viscosity. The viscosity measurements revealed that samples containing soluble dietary fibres, such as pectin, guar, and alginate, exhibited significantly higher viscosities ($p < 0.001$) compared to those with insoluble fibres. Notably, guar exhibited the most pronounced effect, different from alginate (Fig. 3A), with viscosities of 7.65 mPa s for 30 mg and 11.01 mPa s at 90 mg, compared to the viscosity of control being 1.72 mPa s, demonstrating a concentration-dependent



increase. Pectin and alginate (Fig. 3B), displayed similar behaviour, with the viscosity being 2.45 mPa s, for 30 mg pectin and 2.35 mPa s for 30 mg alginate, while being 4.52 mPa s at 90 mg pectin and 4.32 mPa s at 90 mg alginate. In contrast, samples containing insoluble fibres, such as cellulose and RS II even at 90 mg, exhibited viscosities comparable to the control, at 1.71 mPa s and 1.77 mPa s respectively with little variation between different concentrations.

Surface tension

Overall, differences in surface tension between the various digesta were rather small in the present study (below 10%). Both, DF type and doses had a significant impact on the surface tension of the digesta individually ($p < 0.001$), while the interactions between DF type and dosage also turned out to be significant ($p = 0.008$). Guar had the strongest impact on surface tension of the digesta compared to controls, decreasing it by a relative 30% compared to controls ($p = 0.001$), (ESI Fig. 1†), while both pectin ($p = 0.01$) and alginate ($p = 0.01$) showed a relative decrease of 15% vs. controls. The decrease in surface tension was significant at 30 and 90 mg of DF, for guar, pectin and alginate. RS II ($p = 0.03$) showed a relative decrease in surface tension by 11% at 30 mg, but not at 90 mg.

Micelle size

As samples other than alginate and pectin at 90 mg could not be measured for true micelle size (due to the large amount of zero readings), only the difference between these two groups was assessed, using a Mann–Whitney–U test. Results did not reveal any significant difference between micelle sizes. For the remaining groups, Kruskal–Wallis analyses were conducted on size assumed to reflect lipid droplets, with neither the individual nor interactions between DF type and dosage showing any significant impact on size.

ζ -Potential

DF type, their dosage, as well as their interaction, all had a significant impact on the ζ -potential of micelles obtained from digesta, ($p < 0.001$). All ζ -potential measures produced negative readings. The lowest absolute ζ -potential was observed for guar at 30 mg at 31.8 ± 7.1 mV. With respect to dosage, pectin and alginate showed significant differences between 0, 30 and 90 mg dose levels. For pectin the decrease in the absolute ζ -potential was from 50.6 ± 0.2 mV (controls) to 44.6 ± 0.7 mV 30 mg ($p = 0.009$) and further to 37.0 ± 4.3 mV ($p < 0.001$) at 90 mg. Alginate decreased the ζ -potential to 42.6 ± 2.3 mV ($p = 0.011$) at 30 mg and 43.0 ± 5.6 mV ($p = 0.017$) at 90 mg vs. 50.6 ± 0.2 mV (controls). For FOS, a significant decrease in the values was observed exclusively at 30 mg 41.2 ± 7.8 mV ($p = 0.016$), while for RS II at 90 mg 47.9 ± 4.8 mV ($p = 0.027$). Lastly, cellulose did not have a significant impact at any of the dose levels (Fig. 4).

Lipid digestion

Both DF type and dosage had a significant impact on FFA release, and thus on TG lipolysis (both $p = 0.001$) (Fig. 5). DF at

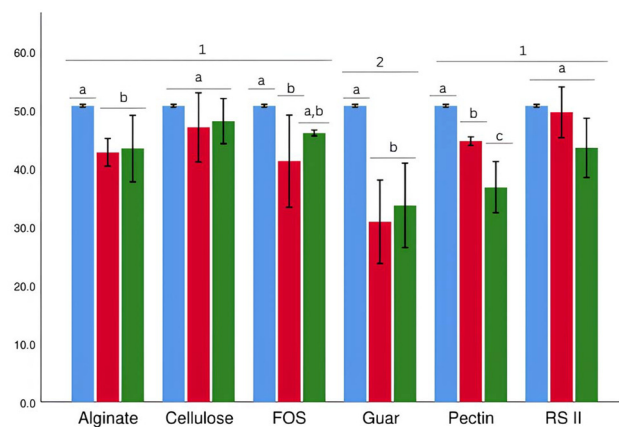


Fig. 4 Bar chart illustrating the mean absolute ζ -potential values (mV) for different fibre types, i.e. alginate, cellulose, resistant starch II (RS II), fructooligosaccharides (FOS), guar and pectin at three doses. Each fibre type is shown at three bars corresponding to different quantities, with (blue) representing 0 mg, (red) for 30 mg, and (green) for 90 mg. Error bars represent standard deviation across three quantities. Both individual factors, fibre type ($p < 0.001$) and quantities ($p < 0.001$) as well as interactions between the two factors ($p = 0.001$) were significant. Guar showed lower values compared to all other fibres, whereas, FOS showed the maximum ζ -potential. All group-wise comparisons were done using Tukey's *post hoc* test, with fibres categorized into subsets 1 and 2. Within each fibre type, different letters (a, b and c) indicate significant differences among the three doses.

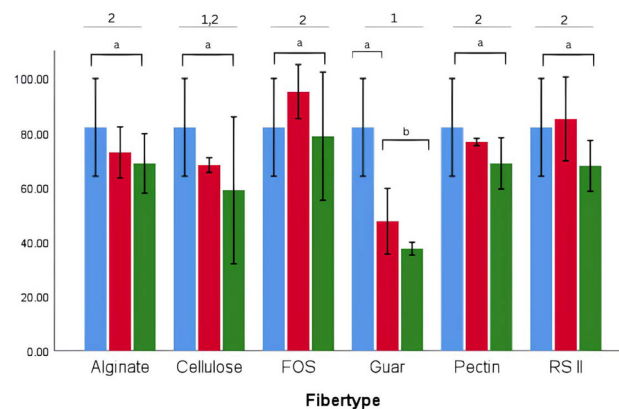


Fig. 5 Percentage cleavage of free fatty acids across different dietary fibre types alginate, cellulose, fructooligosaccharides (FOS), guar, pectin, and resistant starch II (RS II) at three doses. The x-axis represents various dietary fibre types, while the y-axis indicates the mean percentage cleavage of free fatty acids and standard deviation across each condition, where a percentage of 100% would be equivalent of two molecules of fatty acid cleaved per molecule of triglyceride. Error bars represent standard deviation across three quantities. Three different fibre quantities compared were 0 mg (blue), 30 mg (red), and 90 mg (green). Both individual factors, fibre type ($p < 0.001$) and quantities ($p < 0.001$) were significant. All group-wise comparisons were done using Tukey's *post hoc* test, with fibres categorized into subsets 1 and 2. Within each fibre type, different letters (a, b and c) indicate significant differences among the three doses.



90.0 mg had the strongest hampering effect TG lipolysis, irrespective of the DF type compared to controls ($p < 0.001$). The percentage of FFA release decreased from $82.0 \pm 15.8\%$ for controls to $74.1 \pm 17.4\%$ for 30 mg dose and further to $63.3 \pm 19.3\%$ at 90 mg. Taking into account the type of DF (considering pooled quantities), guar had the strongest effect and lowest FFA release $55.6 \pm 22.9\%$, while the mean cleavage percentage for free fatty acids was highest for FOS at $85.2 \pm 17.8\%$.

Correlation of bioaccessibility with physico-chemical properties and lipid digestion

For all carotenoids, bioaccessibility showed a significant negative correlation with viscosity, *i.e.* β -carotene ($r = -0.266$, $p = 0.024$), lutein ($r = -0.268$, $p = 0.023$) and lycopene ($r = -0.420$, $p < 0.001$). Bioaccessibility also exhibited a significant negative correlation with zeta potential for β -carotene ($r = -0.360$, $p = 0.002$), lutein ($r = -0.295$, $p = 0.012$) and lycopene ($r = -0.461$, $p < 0.001$). Moreover, surface tension correlated significantly with bioaccessibility for β -carotene ($r = 0.271$, $p = 0.021$), lutein ($r = 0.245$, $p = 0.038$) and lycopene ($r = 0.369$, $p = 0.001$). While no significant correlation was found between bioaccessibility and FFA release for lutein, a marginal correlation was observed in case of β -carotene ($r = 0.222$, $p = 0.060$); and a significant correlation was seen in case of lycopene ($r = 0.241$, $p = 0.042$).

Discussion

This study explored whether different frequently consumed DF can modulate carotenoid bioaccessibility, a key step in carotenoid bioavailability. Amongst the studied DF, guar, pectin and alginate, *i.e.* soluble, gel-forming DFs, showed negative and rather dose-dependent effects on the bioaccessibility of lutein, β -carotene and lycopene, reducing their relative bioaccessibility by up to 59% compared to DF-free controls.

This study demonstrated that all studied carotenoids were significantly influenced by the type and dose of DF, as well as their interactions, indicating varying effects based on the specific DF and carotenoid. Particularly those DF known for their gel-forming and thickening properties, such as pectin, alginate and guar,^{53,70,71} significantly reduced carotenoid bioaccessibility. In contrast, insoluble fibres such as cellulose⁷² and low solubility RS II^{73,74} as well as FOS, a soluble but non gel-forming DF, had either no negative effect or even slightly enhanced bioaccessibility.

DF could negatively affect carotenoid micellization through changes in the physico-chemical properties of the digesta. Especially soluble fibres with gel-forming ability, can create viscous gels that may hinder enzyme access, limiting digestion and preventing the formation of mixed micelles from lipid droplets or entrapping them, reducing carotenoid bioaccessibility.²⁶ In line, we observed a significant inverse correlation between viscosity and bioaccessibility in our study, especially for guar, alginate and pectin that increased viscosity and reduced surface tension to some degree. On the other hand, the insoluble DF cellulose and RS II are not gel-forming and

hardly impacted surface tension or viscosity of the digesta and carotenoid bioaccessibility. Reduced surface tension was observed rather for soluble fibres, possibly as some lower-molecular compounds present in the DF compounds such as small amounts of other carbohydrates, or the presence of the soluble DF themselves, could have reduced surface tension. However, these findings are in accordance with earlier studies, where cellulose and wheat bran did not negatively impact carotenoid bioavailability.³⁸

Among the gel-forming DF types studied, alginate had the most pronounced effect on β -carotene and lycopene bioaccessibility, reducing it by up to 59% and 43%, respectively. Alginate is a natural anionic linear copolymer, derived from different species of marine brown algae and contains free hydroxyl and carboxyl groups that enable the formation of strong hydrogen bonds, enabling its gel-forming properties.⁷⁵ In the presence of divalent cations such as calcium or magnesium, present during simulated digestion, a strong interaction may occur between these ions and the carboxyl groups of guluronic acids, creating a 3-D lattice insoluble in water, which may entrap lipid droplets or mixed micelles.⁷⁰ In contrast, lutein bioaccessibility was not significantly impacted by alginate. β -Carotene and lycopene are highly hydrophobic, and less easily incorporated into mixed micelles compared to the more hydrophilic lutein. Yonekura and Nagao⁷⁶ revealed detrimental effects of pectin and alginate on micellization of β -carotene and lutein and cellular uptake relative to the fibre-free control. Furthermore, Naumann *et al.*⁷⁷ speculated on a hydrophobic linkage between bile acids and DF, supporting that viscous food matrices may impinge micellization. These findings were corroborated by the lower amounts of FFA released in the presence of alginate in our study, supposedly hampering micellization. In this regard, also micelle size plays a crucial role in determining emulsion stability, with smaller micelles being more stable due to a lower tendency of coalescence.⁷⁸ In the present study, we could only for alginate and pectin at 90 mg doses obtain size measurements reflecting true mixed micelles, in line with increased short-term stabilisation of emulsions upon addition of pectin, inhibiting the aggregation of lipid droplets.⁷⁹ Also another study with oil-in water emulsions improved stability and produced smaller particle sizes with the addition of sodium alginate concentrations ranging from 0.1 to 0.4%.⁸⁰ Another critical factor influencing emulsion stability is the ζ -potential, with a higher absolute potential indicative of stronger repulsion, preventing coalescence,⁸¹ expected to translate into higher micellization, as observed with the control samples, while the addition of guar, pectin and alginate reduced the absolute ζ -potential, indicating less stable micelles, in line with lower observed bioaccessibility. FOS, RS II, and cellulose had a less strong impact.

Pectin is a naturally occurring polysaccharide commonly found in apples, citrus fruits *etc.*, primarily composed of repeating units of α -(1-4)-linked D-galacturonic acid units.⁷¹ Pectin reduced the bioaccessibility of all three carotenoids significantly. This is in accordance with an earlier human study,³⁸ where pectin supplementation decreased the bio-



availability of lutein, lycopene and β -carotene by around 40%. Like alginate, pectin can form 3-D networks. Among other, the degree of methyl esterification (DME), *i.e.* low-methoxyl pectin (LMP, DME < 50%) *vs.* high-methoxyl pectin (HMP, DME > 50%) determines its gelling properties in food applications.^{71,82} The pectin used in this study was an HMP (DME between 50–75%). The acidic environment in the stomach may cause a HMP to form a gel.⁷¹ It was shown that both HMP and LMP can bind calcium, further fostering gelation.⁸³ Studies showed that a minimum amount of calcium is needed for the activity of *e.g.* intestinal lipase, as they may enable access of lipase to emulsified lipids.^{84,85} Thus, pectin gels may lower the amount of available calcium ions, hampering lipase activity. In another study, it was shown that pectin reduced the availability of bile salts,⁸⁶ which may constitute another mechanism by which pectin may reduce carotenoid bioaccessibility.

Guar gum, another soluble DF, extracted from guar beans and another thickening and stabilizing agent⁵³ had a less strong effect on the bioaccessibility of β -carotene and lutein but a more noticeable one on lycopene. Guar, similar to pectin and alginate, increased the viscosity of the digesta, highlighting its strong thickening ability, especially at higher concentrations. Few studies have employed guar gum and mixtures of guar and xanthan to stabilise β -carotene loaded liposome emulsions, indicating a stabilising effect on emulsion stability,^{87,88} in line with a hampered processing of lipid droplets to mixed micelles, as reflected in the reduced triglyceride lipolysis in the present study. Of note, guar, unlike pectin and alginate, is forming less extensive 3-D networks and is more renowned for its thickening properties,⁸⁹ which may explain a somewhat different behaviour on carotenoid bioaccessibility. Although guar had the highest viscosity, lowest ζ -potential, and lowest FFA release, its effect on bioaccessibility was less pronounced compared to pectin and alginate, indicating that additional factors appear to play a role that determine bioaccessibility. As guar is a non-charged molecule (only hydroxyl groups present), this may relate to lowered interferences with the negatively charged mixed micelles compared to alginate and pectin, though this remains speculative. Guar had the strongest inhibitory effect on FFA release, which was at least partially aligned with its negative impact on bioaccessibility and gel-forming properties. However, these findings align with studies highlighting the de-emulsifying effect of guar gum, reducing triglyceride lipolysis and subsequently lipid emulsification.²⁸

FOS is another soluble, though not gel-forming low molecular weight DF commonly found *e.g.* in artichokes, chicory, onions, leeks and asparagus.⁹⁰ In the current study, it did not reduce the bioaccessibility of any of the three carotenoids, in line with only small changes observed regarding TG lipolysis, ζ -potential, viscosity and surface tension. Conversely, FOS did slightly but significantly increase the bioaccessibility of lutein and β -carotene. With a noticeable effect at 30 mg, FOS showed highest cleavage of FFAs followed by RS II, which was well aligned with high bioaccessibility, indicating that FFA may be

a good indicator for bioaccessibility. An improvement in carotenoid bioaccessibility induced by soluble, non-gel forming fibres could, at least in theory, be attributed to stabilizing lipid droplets or preventing the coalescence of mixed micelles,⁹¹ due to increased viscosity or reduced emulsification of lipids,²⁸ or also removal of calcium and other divalent ions that may precipitate free fatty acids required for micellization, though this effect may occur only at high concentrations of divalent ions.^{42,92} More research is needed if such effects are possible by soluble, non-gel forming types of dietary fibre.

Cellulose is a dominant component of the cell wall, composed of linear, ribbon-shaped polymers of glucose.⁹³ The insolubility of cellulose in aqueous systems is attributed to its rather crystalline-like structure. In the present study, cellulose did not negatively affect carotenoid bioaccessibility, and even increased it slightly for all three carotenoids. Similar as for FOS, it did not have an important impact on surface tension, viscosity, or lipid digestion.

Finally, RS II, a native crystalline ungelatinized starch found commonly in green bananas and raw potatoes,⁹⁴ behaved similar to cellulose, not exhibiting a significant impact on carotenoid bioaccessibility, rather showing a minor increase in bioaccessibility of lutein and lycopene. No significant impact was noted on lipid digestion, ζ -potential, surface tension and viscosity.

To conclude, this study revealed that the type and dosage of DF substantially impacted the bioaccessibility of carotenoids, with especially soluble, gel-forming fibres such as pectin and alginate and to a lesser extent guar exhibiting the most detrimental impact on carotenoid bioaccessibility. This negative impact on bioaccessibility correlated with hampered enzymatic lipid digestion and reduced absolute ζ -potential. Non-gel-forming fibres, *i.e.* cellulose, RS II, and FOS, demonstrated no negative and even slightly positive effects on carotenoid bioaccessibility. Further studies should aim to clarify further mechanistic aspects and more complex interactions, such as the presence of other dietary components during digestion, as expected for real food matrices and their impact on aspects of carotenoid bioavailability.

Abbreviations

AI	Adequate intake
AMD	Age-related macular degeneration
AUC	Area under the curve
CVD	Cardiovascular diseases
DA	Degree of acetylation
DE	Degree of esterification
DF	Dietary fibre
EFSA	European Food Safety Authority
FFA	Free fatty acid
FOS	Fructooligosaccharides
GI	Gastrointestinal
HMP	High methoxyl pectin
LMP	Low methoxyl pectin



LSD	Least significant difference
RS II	Resistant starch type-II
SD	Standard deviation
SGF	Simulated gastric fluids
SIF	Simulated intestinal fluids
TG	Triglycerides

Conflicts of interest

There are no conflicts of interest to declare.

Data available statements

The raw *in vitro* data supporting this article have been included as ESI.†

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