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Magali Gies[a,b], Adrien Servent[a,b], Patrick Borel[c], Claudie Dhuique-Mayer[a,b*]

[a] CIRAD, UMR Qualisud, F-34398 Montpellier, France

[b] Qualisud, Univ. Montpellier, CIRAD, Montpellier SupAgro, Université d'Avignon, Université de La Réunion, Montpellier, France

[c] C2VN, INRA, INSERM, Aix Marseille Univ, Marseille, France

[*]Corresponding author: claudie.dhuique-mayer@cirad.fr

Abstract

Functional foods containing dispersible phytosterols (DPs) are recommended to reduce cholesterol absorption in humans. However, only a few studies have been conducted on the effect of DP vehicles on carotenoid bioaccessibility and uptake by intestinal cells. On a previously developed fermented maize product containing both DPs and carotenoids, we aim to evaluate the effect of DPs encapsulated by maltodextrin on the carotenoid/phytosterol/cholesterol interactions during gastro-duodenal digestion. Thanks to maltodextrin properties, β-carotene and lycopene bioaccessibilities were significantly improved by 40.2 ± 1.4% while that of β-cryptoxanthin decreased by 14 ± 0.1%. Additionally, the presence of DPs reduced the cholesterol micellarization by 51.8 ± 4.2%. Despite a slight decrease in carotenoid uptake caused by the DP vehicle, related to micelle size, an inhibition of cholesterol accumulation by Caco-2 cells was observed. These results highlighted the key role of maltodextrin as a vehicle of DPs on carotenoid and cholesterol absorption.

Keywords

Dispersible phytosterols; maltodextrin; carotenes; xanthophylls; bioaccessibility; Caco-2 cells

1. Introduction

Phytosterols are frequently used in the functional food industry because they are known to be effective in lowering the LDL-cholesterol level (Gylling & Simonen 2015). Several dairy products, such as yogurts or margerines, are enriched in phytosterols and have been on the market for several years (Descalzo *et al.*, 2018). The daily recommended intake of phytosterols to obtain a cholesterol-lowering effect is 1.5 to 3 g. It allows reducing the cholesterol absorption from the gut by 30-40% leading to 7-12% decrease of plasma LDL-cholesterol (Chen, McClements, & Decker, 2013; EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA), 2012; Marangoni & Poli, 2010; Shahzad *et al.*, 2017). Recently, it was explained that functional foods with plant sterols may be considered in hypercholesterolemic patients with intermediate and low global cardiovascular risk (Gylling *et al.*, 2014).

Phytosterols are natural constituents of plant cell walls. Among the 250 identified phytosterol molecules, β-sitosterol is the most abundant phytosterol along with campesterol and stigmasterol. Because of the structural similarities between sitosterol and cholesterol (an additional ethyl group at position C-24 in sitosterol), it is known that sitosterol can reduce cholesterol absorption. The two main mechanisms involved in the cholesterol-lowering effect of phytosterols are : their competition with cholesterol molecules during the incorporation into mixed micelles in the digestive tract ; and the co-cristallization with cholesterol, leading to an increase in the fecal excretion of cholesterol (Marangoni & Poli 2010). Another mechanism described the competition between cholesterol and phytosterols for uptake by intestinal transporters (NPC1L1 and SR-BI) and a biliary secretion due to an apical efflux of phytosterols from the enterocytes via ABCG5/G8 (Nakano, Inoue, & Murakoshi, 2019). Moreover, the *Trans-Intestinal Cholesterol Excretion* (TICE) was described as a complementary mechanism of the biliary secretion, inversely correlated with the development of atherosclerosis (Blanchard, Moreau, Cariou, & Le May, 2014).

Data suggest that the type of phytosterols (free or esterified) and the food matrix can influence the interaction between phytosterols and cholesterol (Gleize *et al.*, 2016). Solubilized sterols are more often used in low-fat dairy products than sterol esters. They also induced a similar LDL-cholesterol lowering effect (Thomsen *et al.* 2004; Shaghaghi, Harding, & Jones 2014). Bohn *et al.* (2007) showed

that fat-free phytosterol-containing products were able to significantly decrease cholesterol micellarization and Caco-2 cell absorption, depending on the formulation of products. Moreover, while micro-encapsulation is known to enhance the stability of compounds and their bioaccessibility (Kopec & Failla, 2018), Spilburg *et al.* (2003) demonstrated that powdered soy stanol-lecithin used as emulsifier decreased cholesterol absorption and LDL cholesterol when consumed in fat-free foods.

However, a major concern is the interaction between phytosterols and fat-soluble bioactive compounds, like carotenoids or tocopherols, during digestion (Noakes *et al.*, 2002; Richelle *et al.*, 2004; Rudkowska, AbuMweis, Nicolle, & Jones, 2008). Indeed, recent meta-analyses reported the decrease of carotenoid concentrations in plasma after plant sterol consumption (Baumgartner *et al.* 2017; Fardet *et al.* 2017).

Consequently, it is recommended to increase the consumption of fruits and vegetables rich in carotenoids together with the consumption of functional sterol products (Fardet *et al.* 2017). Carotenoids are known to be a natural antioxidant (such as lycopene (Lyc)), but also enhancers of the immune system, decreasing the risk of cardiovascular and eye diseases (Jomova & Valko, 2013). Some of them are indeed provitamin A carotenoids (β-carotene (Bc), α-carotene, β-cryptoxanthin (Bcx)) and have many vital systemic functions after bioconversion into vitamin A in the organism (Fernández-García *et al.*, 2012). The lipophilic nature of carotenoids could explain the decrease in their absorption when phytosterols are present. Apolar Bc is solubilized within mixed micelles whereas Bcx, like xanthophyll, is probably located on the surface of lipid droplets (Borel *et al.*, 1996). Therefore, phytosterols can replace not only cholesterol in the core of mixed micelles but also other fat-soluble compounds, such as carotenoids (Baumgartner *et al.*, 2017). However, both esterified or free phytosterols are known to compete with carotenoids for absorption during gastro-duodenal digestion, in a dose-dependent manner, even if it is less with free phytosterols (Richelle *et al.*, 2004). That is why it is necessary to supplement a functional product containing phytosterols with carotenoids, to reach at least 2 mg per 125 g serving portion (Noakes *et al.*, 2002).

In this context, our laboratory developed a new probiotic functional food based on fermented maize, containing dispersible phytosterols (DPs) and enriched with natural carotenoids from papaya and melon. This yogurt like-product was designed to be an alternative to current functional dairy products (Gies, Descalzo, Servent, & Dhuique-Mayer, 2019). Due to possible interactions between phytosterols

and fat-soluble compounds for micellarization, the bioaccessibility of carotenoids and dietary cholesterol of this new functional food had to be assessed, taking into account the role of the vehicle of DPs. Indeed, to the best of our knowledge, there is no study on the impact of maltodextrin as vehicle of DPs on carotenoid bioaccessibility. Specifically, our aims were 1) to evaluate the bioaccessibility of carotenoids with and without added DPs micro-encapsulated with maltodextrin and 2) to understand if these DPs decrease cholesterol micellarization during *in vitro* digestion of the fermented yogurt-like product with added cholesterol, in order to validate the cholesterol-lowering effect of these specific DPs. In the second part, fat-soluble cellular uptake was measured using *in vitro* digestion coupled with the Caco-2 cell culture model.

2. Material and methods

2.1. Fermented maize yogurt-like product

This functional product was previously developed by our laboratory to obtain a probiotic yogurt-like food, based on fermented maize, designed to be functional. Indeed, it was enriched with carotenoids and DPs to confer a cholesterol-lowering effect and provide a high intake of provitamin A. The material contains 2.05 mg of carotenoids/portion of 125 g, which is nearly half of the estimated daily carotenoid consumption in several countries of 1.3 - 5.84 mg (Gies, Descalzo, Servent, & Dhuique-Mayer, 2019; Toti *et al.*, 2018). The manufacturing process included maize soaking, crushing, sieving, pasteurization and lactic fermentation. DPs and freeze-dried fruits (papaya and melon) were incorporated before the pasteurization step (Gies, Descalzo, Servent, & Dhuique-Mayer, 2019). Commercial DPs, S-80 WDP 90% non-GMO with 80% β-sitosterol were provided by Vitaesterol® (Vitae Naturals, Spain). This type of DP is spray-dried with 10% maltodextrin (16.5-19.5 dextrose equivalent) and 3% sucroesters, in order to ensure good encapsulation and therefore their hydrophilic solubility.

The product was formulated either with or without DPs, and with maltodextrin with the same dextrose equivalent, or with maltodextrin and sucroesters. Interaction mechanisms during digestion were studied thanks to products with added standards of β-sitosterol (Supelco/Sigma-Aldrich, USA) and cholesterol (Sigma-Aldrich, France). The final products were kept frozen and in darkness at -20 °C until analysis.

2.2. Fat-soluble compound analysis

The carotenoids, phytosterols and tocopherols of the yogurt-like product were analyzed by a single analysis method UPLC-DAD-FLD with an UPLC –1290 System Infinity II (Agilent, USA) according to Gies, Descalzo, Servent, & Dhuique-Mayer (2019). Briefly, 1 g of product was saponified and extracted twice with n-hexane. Hexanic phases were evaporated under nitrogen and dissolved in 1 mL of a MTBE/methanol solution (4:1, v: v) before injection into the UPLC system.

Carotenoid extraction from digested samples was performed as previously described by Dhuique-Mayer *et al.* (2018). An aliquot of 10 mL of the micellar phase from a digested sample was extracted 3 times with 10 mL of hexane and 5 mL of ethanol containing 150 µL of β-apo-8'-carotenal as an internal standard. The pooled hexane extracts were evaporated and dissolved in 500 µL of MTBE/methanol (4:1; v:v). Samples were injected according to the UPLC conditions described below.

The column used was a C30 YMC (150 x 4.6 mm; 3 µm) (YMC Europe GMBH, Germany). Mobile phases were methanol as eluant A, water as eluant B and MTBE as eluant C, set at 1.5 and 1 mL/min flow rate for carotenoids/phytosterols/tocopherols and cholesterol respectively. The gradient used to separate carotenoids, phytosterols and tocopherols was the following: 0-1.5 min [60% A, 40% B]; 1.5-3 min [80% A, 20% B]; 3-12.5 min [80% A, 5% B, 15% C]; 12.5-15 min [15% A, 85% C]; 15-17 min [100% A] and back to the initial conditions for re-equilibration. The other gradient used for cholesterol was: 0-1 min [60% A, 40% B]; 1-1.5 min [80% A, 20% B]; 1.5-3 min [80% A, 5% B, 15% C]; 3-25 min [65% A, 5% B, 30% C]; 25-27.7 min [15% A, 85% C]; 27.7-30 min [100% A] and back to the initial conditions for re-equilibration. The column temperature was 20 °C and the injection volume was set between 10 µL and 40 µL. Detection was set at 210 nm (DAD) for phytosterols and cholesterol, 450 and 470 nm (DAD) for carotenoids. Fluorescence detection (FLD) for tocopherols was set at 296 nm (excitation) and 330 nm (emission). Quantification was achieved using the external calibration curve of Bc, β-cryptoxanthin, lycopene, α/γ-tocopherol standards (Extrasynthese, France) and β-sitosterol standard (Supelco, USA).

2.3. In vitro digestion model

The *in vitro* digestion model was previously developed by Reboul *et al.* (2006). It had been validated against human studies and was considered to be a reliable model for carotenoid behavior during *in vitro* digestion (Etcheverry, Grusak, & Fleige, 2012). 15 g of product samples were mixed in 32 mL of saline solution (NaCl 0.9%) and submitted to the *in vitro* digestion model according to Dhuique-Mayer *et al.* (2016). To mimic the gastric digestion phase, the pH was adjusted to 4.0 and 2 mL of pepsin were added before incubating the mixture at 37 °C for 30 min. To mimic the duodenal phase, the pH of the gastric mixture was raised to 6.0. Then, 9 mL of a solution containing porcine pancreatin (2 mg/mL) and porcine bile extract (12 mg/mL) in 100 mmol/L trisodium citrate were added, as well as 4 mL of porcine bile extract (0.1 g/mL) and 1 mL of cholesterol esterase (10 mg 32 U/mL in 6 mL of distilled water). Samples were subsequently incubated in a shaking water bath at 37 °C for 30 min to finish the digestion process. Micelles were separated by centrifugation at 48 000 g for 4 h at 10 °C using an Aventi JE rotor JA-20 (Beckman-coulter, USA), and the aqueous fraction was collected and filtered through a 0.20 µm filter (Whatman, U.K.). Aliquots were stored at -20 °C until analysis.

The maltodextrin and the sucroesters (as used in the commercial DPs with 10% maltodextrin and 3% sucroesters) were added to 15 g of the product before starting the *in vitro* digestion process. Maltodextrin (dextrose equivalent 16.5-19.5) was purchased from Sigma-Aldrich (France), while sucroesters (E473) were purchased from Louis François (France). β-sitosterol or cholesterol standards (Sigma-Aldrich, France) were solubilized in tetrahydrofuran (THF) at 0.25 g/mL and 0.05 g/mL respectively. These solutions were also added into the 15 g of product before *in vitro* digestion, according to each experiment, to reach a concentration of β-sitosterol necessary to obtain a cholesterol-lowering effect, and to simulate the digestion of the cholesterol content of 5 g of fat food (based on pork rillettes in this study).

2.4. Mixed micelle size

The size of mixed micelles from the micellar phase was measured at 25 °C using the photon correlation spectroscopy analysis (Zetasizer Nano-ZS, Malvern Instruments, UK).

2.5. Measurement of fat-soluble compound uptake by intestinal cells

Caco-2 clone TC7 cells were a gift from Dr. M. Rousset (U178 INSERM, Villejuif, France). Culture cell conditions were performed according to Gence *et al.* (2018) with minor modifications. Briefly, cells were maintained in DMEM supplemented with 20% heat-inactivated fetal bovine serum, 1% non-essential amino acid, 1% streptomycin, and 2% L-glutamine. For each experiment, cells were sowed at a density of $5x10^5$ cells/25 cm^2 flask (Becton Dickinson, le Pont-de Chaix, France) for 21 days to obtain confluent differentiated cell monolayers. Carotenoid-rich micelles derived from the *in vitro* digestion were diluted at 1:4. At the beginning of each experiment, cell monolayers were washed with 2 mL of phosphate buffered saline (PBS). Cell monolayers were incubated with 7 mL of diluted micelles for 2h at 37 °C. Media and cells were collected after the incubation period. Cell monolayers were collected in 2 mL of PBS. Note that for cholesterol measurement, a control without any incubation of micelles was used as a reference. All samples were stored at -80 °C under nitrogen before carotenoid extraction. HPLC analysis was carried out as reported by Gence *et al.* (2018). Cholesterol and β-sitosterol were detected at 210 nm and identified by comparaison with pure standards.

2.6. Statistical analyses

Data were analyzed statistically using one-way analysis of variance (ANOVA) in order to determine significant differences ($p < 0.05$). Tukey's multiple comparison method was used as a post-hoc test to further compare means together.

3. Results and discussion

3.1. Fat-soluble compound content of the fermented maize yogurt-like product

Carotenoid, tocopherol and β-sitosterol contents of the fermented yogurt-like product are reported in Table 1. The three main carotenoids, which were Bcx, Bc and Lyc, came from papaya and melon extracts. They represented a total carotenoid content of 26.3 ± 0.8 mg/kg, Bc being the major provitamin A carotenoid. α-tocopherol and γ-tocopherol (provided by maize and fruits) content were of 3.8 ± 0.1 mg/kg. This is equivalent to 4% of the Recommended Daily Allowance, which is low, but valuable for their antioxidant role, both *in vitro* for the product stability and *in vivo* for the organism. The added DPs, represented 1.68 g/100 g available portion, the same as the minimum recommended

intake of 1.6 g/day to display a cholesterol-lowering effect (Chen, McClements, & Decker, 2013; Marangoni & Poli, 2010; Shahzad *et al.*, 2017). *In fine*, this product provided a functional balance between carotenoids and phytosterols, necessary to induce a cholesterol-lowering effect without a detrimental effect on carotenoids (Noakes *et al.*, 2002)

3.2. Bioaccessibility of fat-soluble compounds in the fermented maize yogurt-like product

The bioaccessibility of β-cryptoxanthin, β-carotene, lycopene, β-sitosterol was studied in this product (Figure 1). Carotenoid bioaccessibilities were ranked as follows: 29.9 ± 3.0% (Bcx), 28.0 ± 0.5% (Bc) and 5.5 ± 0.5% (Lyc), according to this order Bcx ≥ Bc > Lyc in agreement with the literature (Kopec & Failla, 2018). Indeed, it is known that efficiency of carotenoid micellarization is positively correlated with their hydrophilicity. Because of their presence in the food matrix, tocopherol bioaccessibility was also evaluated. Since their bioaccessibility was high (94-100%), interactions with other fat-soluble compounds were unlikely. Although tocopherol bioaccessibility could be variable among dietary sources and processes, the present study was in agreement with previous studies (Reboul *et al.* 2006; Granado-Lorencio *et al.* 2007; Cilla *et al.* 2012).

It is interesting to underline that the β-sitosterol bioaccessibility was very low, with only 1.6 ± 0.3%. This result suggested that a very slight fraction of β-sitosterol added in the formulation was micellarized. This result was confirmed by measuring the β-sitosterol content in the micellar phase, the aqueous phase and the pellets after *in vitro* digestion. It represented 0.22 mg/mL; 2.56 mg/mL and 31.4 mg/mL respectively in these fractions.

It was supposed that a high amount of β-sitosterol was not micellarized because of the vehicle of DPs (mainly maltodextrin). Anyway, in this case, this low β-sitosterol micellarization (1.6%) represented approximatively 34 mg of bioaccessible β-sitosterol/125 g serving portion, sufficient to obtain a cholesterol-lowering effect. Alemany *et al.* (2013) and Garcia-Llatas, Cilla, Alegría, & Lagarda (2015) also used microencapsulated phytosterols and observed low micellarization (from 2.9 to 6.4%) of the added phytosterols in milk-based fruit beverages. These authors reported that the presence of Bcx seemed to decrease the sitosterol bioaccessibility in milk-based fruit beverages, supposing an interaction between carotenoids and phytosterols for incorporation into mixed micelles. Moreover, Alvarez-Sala *et al.* (2016) underlined that the bioaccessibility of phytosterols was better for a product

containing whey proteins with milk fat globule membrane or soy lecithin than for a product containing extra olive oil and soy lecithin. They finally highlighted that the bioaccessibility of phytosterols was influenced by the type and the quantity of fat and emulsifiers used in the formulation. Blanco-Morales *et al.* (2018) showed that the addition of 5.0 g of galactooligosaccharides did not affect the phytosterol bioaccessibility, but could slightly increase the bioaccessibility of cholesterol. Differences between micro or nano-encapsulation and types of carriers of bioactive encapsulated compounds could have an influence on fat-soluble compound bioaccessibility (Soukoulis & Bohn, 2018).

3.3. Effect of DPs and their vehicles on carotenoid bioaccessibility

In order to better understand how these DPs influence carotenoid bioaccessibility, four products were compared: two products, with and without DPs, and two products with the vehicles of encapsulation mainly represented by maltodextrin, either with maltodextrin alone at 10% or maltodextrin associated with sucroesters at 10%-3% (Figure 2A and 2B).

Surprisingly, the bioaccessibility of both Bc and Lyc significantly ($p < 0.05$) increased by 40.2 ± 1.4% in the presence of added DPs as represented on Figure 2. Similarly, carotene bioaccessibility also increased in the presence of maltodextrin alone. Maltodextrin is a polysaccharide produced from partial hydrolyzed starch and currently used as an encapsulating agent of fat-soluble compounds (Medeiros *et al.*, 2019). It is used during the spray-drying of high carotenoid content extracts, and it is known to have properties such as low viscosity and coating capacity, which can also stabilize emulsions (Parikh, Agarwal, & Raut, 2014). Some authors reported that bioaccessibilities of spray-dried carotenoids encapsulated with maltodextrin increased significantly (Kyriakoudi & Tsimidou, 2018; Montero, Calvo, Gómez-Guillén, & Gómez-Estaca, 2016). Therefore, in our study, maltodextrin, added with DPs, significantly increased the bioaccessbility of carotenes.

Conversely, the bioaccessibility of Bcx, of which the concentration was low so it represented a minor provitamin A carotenoid in this product, decreased by 19 and 24% when DPs or maltodextrin respectively were added. This different behavior between carotene and xanthophyll (oxygenated forms) bioaccessibility was probably due to xanthophyll hydrophilicity. It is thought that xanthophylls (as Bcx) are located on the surface of the lipid droplets whereas apolar carotenes (such as Bc or Lyc)

258 are solubilized in the core of lipid droplets (Borel *et al.*, 1996). Maltodextrin and its stabilizing
259 properties appeared to have a lower effect on Bcx bioaccessibility than on Bc, which is less polar.
260 However, the bioaccessibility of carotenoids in the product with the maltodextrin alone were not
261 significantly different ($p > 0.05$) from the bioaccessibility of carotenoids in the product containing DPs.

262 The same experiment on the product without DPs but containing maltodextrin associated with
263 sucroesters, reduced the vehicle effect for Bcx but maintained it for carotenes. The addition of
264 sucroesters seemed to reduce the difference between carotenoid bioaccessibility from the product with
265 DPs and that from the product with vehicles (Figure 2B). Therefore, it seemed that sucroesters, which
266 are known to decrease carotenoid bioaccessibility, counteracted the maltodextrin effect (Grune *et al.*,
267 2010). This second experiment didn't reveal any significant difference ($p > 0.05$) between
268 bioaccessibility of carotenoids in the product containing DPs and the product with the encapsulation
269 excipients. Together, these results suggested that the bioaccessibility of carotenes in the product with
270 DPs was enhanced by the vehicles of DPs used for encapsulation (mainly maltodextrin). Indeed, the
271 competition for absorption during digestion between carotenes and phytosterols seemed to be avoided
272 in these conditions.

273 Maltodextrin is known to be a fat-replacer in the food industry and can be used as a fat-like gel
274 (Hofman, van Buul, & Brouns, 2016). It was thus possible that maltodextrin could enhance the
275 micellarization of the most apolar carotenoids. After digestion of the product, the average diameter of
276 particle size in the aqueous phase was of 51 nm while the mean diameter of the product without DPs
277 measured 189 nm. These results suggested that maltodextrin could contribute to the formation of
278 nanoemulsion systems with oil-in-water interfaces, efficient in enhancing carotenoid solubility or
279 bioaccessibility (Liang *et al.*, 2013).

280 To better understand the role of the vehicles and their interaction between DPs and carotenoids, *in*
281 *vitro* digestion of the product with added free β-sitosterol, without encapsulation, was investigated.
282 Figure 3 shows that the bioaccessibility of Bcx and Bc decreased by 32 and 23% respectively when
283 the product contained free β-sitosterol. Therefore, free β-sitosterol affected the micellarization of Bcx
284 and Bc, while Lyc micellarization was not impacted. Interestingly, the bioaccessibility of free β-
285 sitosterol was 11.8% compared to the dispersible β-sitosterol (1.6%). This last result showed that β-
286 sitosterol without an excipient was more easily micellarized, which induced a detrimental effect on all
287 types of carotenoid bioaccessibility. This is in agreement with several human studies reporting that

288 free or esterified (emulsion form, associated with lipids) phytosterols decreased plasma concentrations
289 of carotenoids and/or tocopherols (Noakes *et al.*, 2002; Richelle *et al.*, 2004).

290 ***3.4. Impact of DPs on cholesterol micellarization during in vitro digestion***

291 In order to assess the effect of DPs on cholesterol micellarization, the yogurt was submitted to *in vitro*
292 digestion with added cholesterol (Figure 4). The reduction in the micellarization of cholesterol was
293 more than 50%. DPs used in our formulation had therefore a potential cholesterol-lowering effect.
294 Beside this effect, carotene micellarization was not affected by DPs whereas the bioaccessbility of Bcx
295 decreased from 28% to 18%. Although Bcx bioaccessibility was also affected by DPs in presence of
296 cholesterol, it represented only 7% of the total carotenoid content of our product and was half
297 provitaminic A. Nevertheless, using *in vitro* and *in vivo* studies, Granado-Lorencio *et al.* (2011) showed
298 that Bcx bioavailability was not affected by the presence of water-dispersible microencapsulated
299 phytosterols, but the vehicle of encapsulation was not mentioned. Conversely, Hernández-Alvarez *et*
300 *al.* (2016) observed that the bioaccessibility of Bcx decreased while phytosterol bioaccessibility
301 increased after the *in vitro* digestion of a dairy beverage enriched in milk fat globule membrane. They
302 highlighted the competitive effect between Bcx and phytosterols for incorporation into micelles.
303 However, they did not find a similar trend in human serum. Additionally, in a recent human study, Bcx
304 seemed to enhance the cholesterol-lowering effect of DPs and to improve the lipid profile (Granado-
305 Lorencio *et al*. 2014).

306

307 A recent meta-analysis study concluded that plant sterol intake decreased plasma concentrations of
308 carotenoids and affected oxygenated carotenoids in different ways (Baumgartner *et al*., 2017). In this
309 review based on 41 trials, Bcx content in plasma was impacted less than the carotene ones,
310 underlining their different behaviors.
311 In our study, we observed the opposite behavior for Bcx, because this carotenoid was more affected
312 by the presence of DPs. However, in these meta-analyses data, the vehicles of the DPs were not
313 considered or discussed, and it could be a critical point influencing carotenoid absorption.
314 Nevertheless, Shaghaghi, Harding, & Jones (2014) highlighted that DPs did not affect fat-soluble
315 vitamins or carotenoids in plasma of moderately hypercholesterolemic subjects; the vehicle used was
316 a polysorbate emulsifier and the carotenoids tested were carotenes and lutein but not Bcx. Note that *in*

317 *vivo* studies reflect carotenoid serum levels, while *in vitro* studies analyze carotenoid bioaccessibility
318 by evaluating the available fraction for enterocytes. Thus, our results underlined the essential role of
319 the phytosterol vehicles incorporated in a functional food. Therefore, the cholesterol-lowering efficacy
320 of DPs in this product was demonstrated, without a detrimental effect on carotenes.

321

322 ***3.5. Carotenoids and β-sitosterol/cholesterol uptake by Caco-2 cells exposed to micelles***
323 ***generated from in vitro digestion***

324 To investigate any potential effect of DPs on intestinal carotenoid uptake, differentiated Caco-2 cells
325 were exposed to micelles generated from *in vitro* digestion of the products with and without DPs. The
326 provitamin A carotenoids, Bcx and Bc, were preferentially absorbed with an average of 16% vs 7.2%
327 respectively in comparison to Lyc (Table 2). Moreover, carotenoid uptake was significantly higher ($p <$
328 0.05) with micelles issued from *in vitro* digestion of the product without DPs. These results can be
329 explained by the relationship between uptake and micelle size. Indeed, the size of mixed micelles
330 formed during the *in vitro* digestion experiments was measured (Table 3). The presence of the
331 vehicles (maltodextrin/sucroesters) with or without phytosterols significantly ($p < 0.05$) modified the
332 size of the micelles. Thus, micelles without phytosterols presented a mean radius (8.33 nm) smaller
333 than those with the vehicles (9.41 nm for P and 10.61 nm for P without DP). The smaller-sized
334 micelles seemed to be taken up better by intestinal cells. Moreover, the higher uptake of carotenoids
335 obtained from digestion of the product without phytosterols could be explained by a mechanism
336 involved in the regulation of intestinal transporters. Indeed, the uptake of carotenoids and phytosterols
337 by enterocyte could be facilitated by the same transporter such as NPC1L1 or SR-BI and
338 consequently, carotenoids could compete with phytosterols for binding to these transporters (Gleize *et*
339 *al.*, 2016; Nakano, Inoue, & Murakoshi, 2019; Reboul, 2013). We performed the same experiments
340 with the addition of cholesterol in order to observe the ability of DPs to reduce cholesterol
341 accumulation by intestinal Caco-2 cells. A slight decrease of cholesterol uptake was observed when
342 DPs were present in mixed micelles (~2%). However, in our study, the ratio of cholesterol to plant
343 sterol was 1:2.5 while the ratio used in human studies is near to 1:5 corresponding to a dietary
344 cholesterol intake of 300-400 mg/day (Bohn *et al.*, 2007). The supplementation of 1.5-2g of

phytosterols reduced by 7% the cholesterol intake (300 mg) in human studies. Finally, a decrease was observed for the uptake of β-sitosterol when cholesterol was present, from 7.9% to 5.8%.

4. Conclusion

This study resulted in a better understanding of the role of DP vehicles on carotenoid bioaccessibility and uptake by Caco-2 cells of a functional cereal-fermented food. Our results showed that carotene bioaccessibility -or solubilization in aqueous phase during digestion- was improved by the vehicle of DPs, i.e. maltodextrin associated with sucroesters. Moreover, these DPs, incorporated in the functional fermented food, were able to induce a potential cholesterol-lowering effect by decreasing cholesterol micellarization without having a detrimental effect on carotene bioaccessibility. Despite a slight decrease in carotenoid uptake caused by the DP vehicle related to micelle size, an inhibition of cholesterol accumulation by Caco-2 cells was observed. Together, our findings underlined the key role of the vehicle of the phytosterols on carotenoid micellarization and Caco-2 cell uptake. Our results supported the use of DPs encapsulated in maltodextrin associated with sucroesters to design functional foods containing natural carotenoids. However, further studies are needed to clarify the specific behavior of Bcx in the interaction with phytosterols in different food matrices. Finally, dietary intervention studies are needed to validate the efficiency of this functional fermented product in humans, mainly the *in* vivo cholesterol-lowering effect without having a detrimental effect on provitamin A carotenoid bioavailability.

Conflict of interest

The authors declare no conflict of interest.

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Figure captions

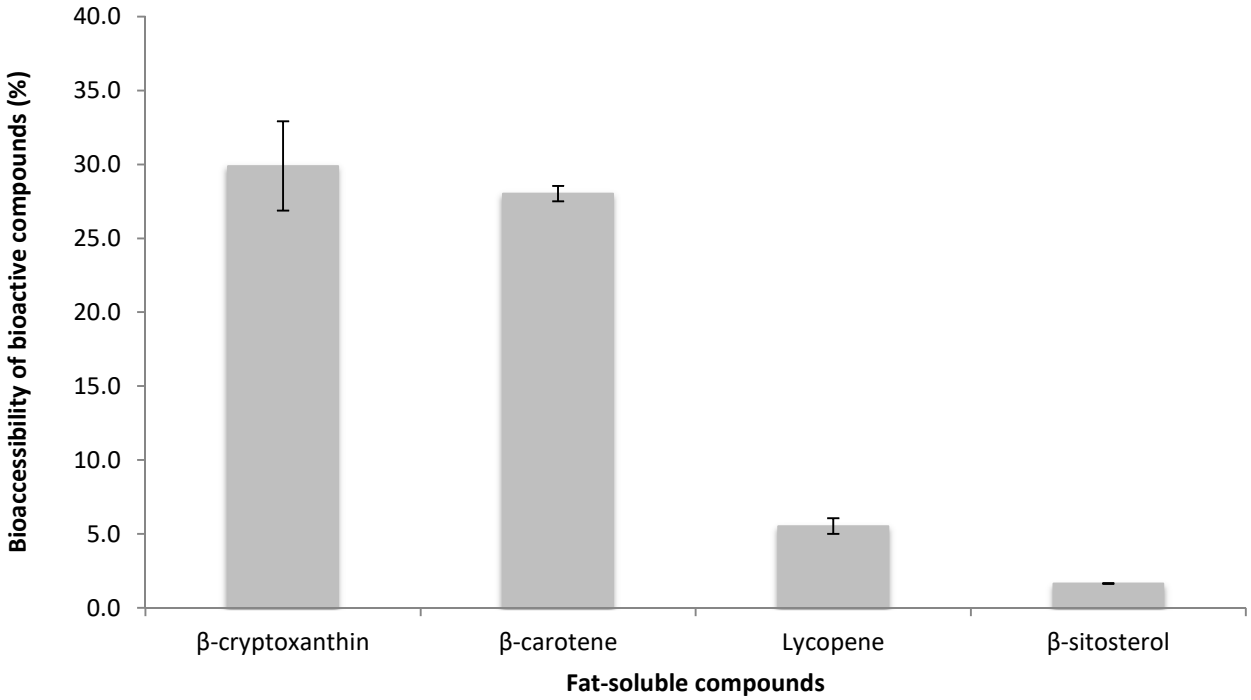
Figure 1: Bioaccessibility of fat-soluble compounds in the functional product

Figure 2: A) Carotenoid bioaccessibility of the functional Product (P) with and without Dispersible Phytosterols (+/-DP) and with maltodextrin vehicule (+m) B) Carotenoid bioaccessibility of the functional Product (P) with and without dispersible phytosterols (+/-DP) and with maltodextrin and sucroester vehicule (+m+s)

Figure 3: Carotenoid bioaccessibility of the functional Product (P) without Dispersible Phytosterols containing standard β-sitosterol; ■ P-DP; ■ P-DP +Bsito

Figure 4: Carotenoid bioaccessibility of the functional Product (P) with or without Dispersible Phytosterols in presence of standard cholesterol; ■ P-DP +chol; ■ P+DP +chol

Figure 1



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529 **Figure 2**

530 **A**

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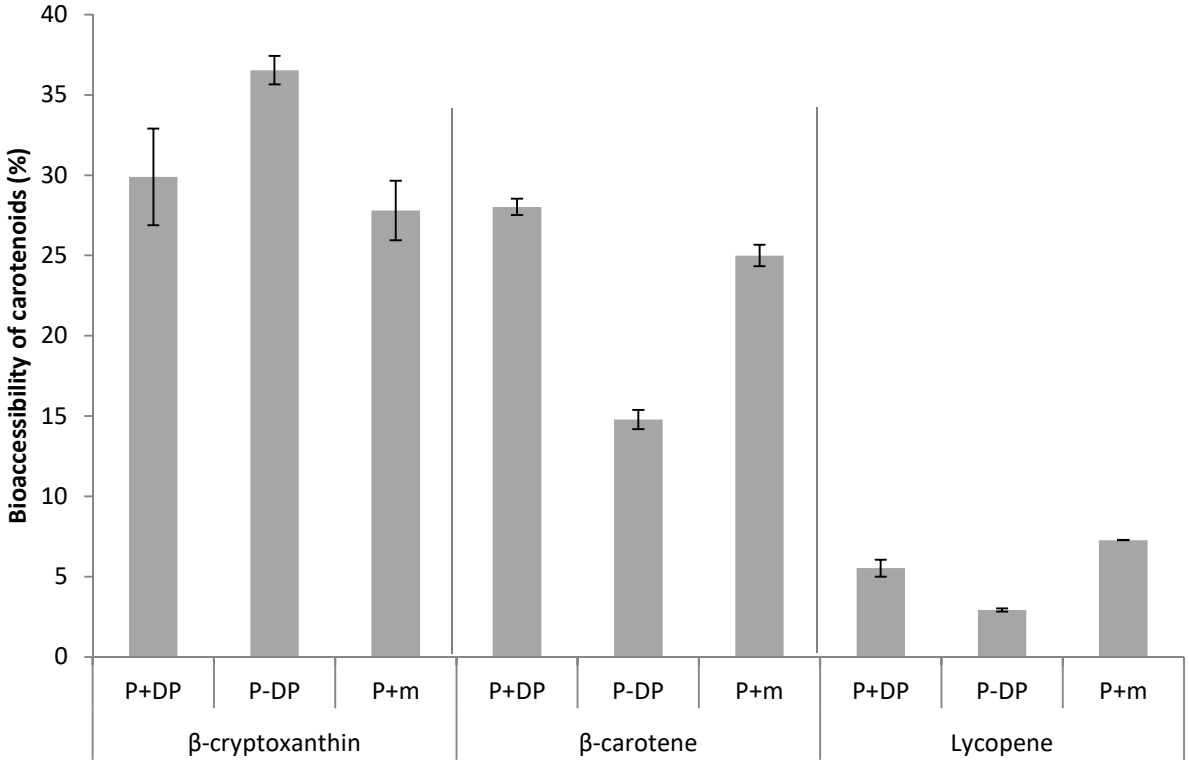
Bioaccessibility of carotenoids (%)
40
35
30
25
20
15
10
5
0
P+DP
P-DP
P+m
P+DP
P-DP
P+m
P+DP
P-DP
P+m
β-cryptoxanthin
β-carotene
Lycopene

B

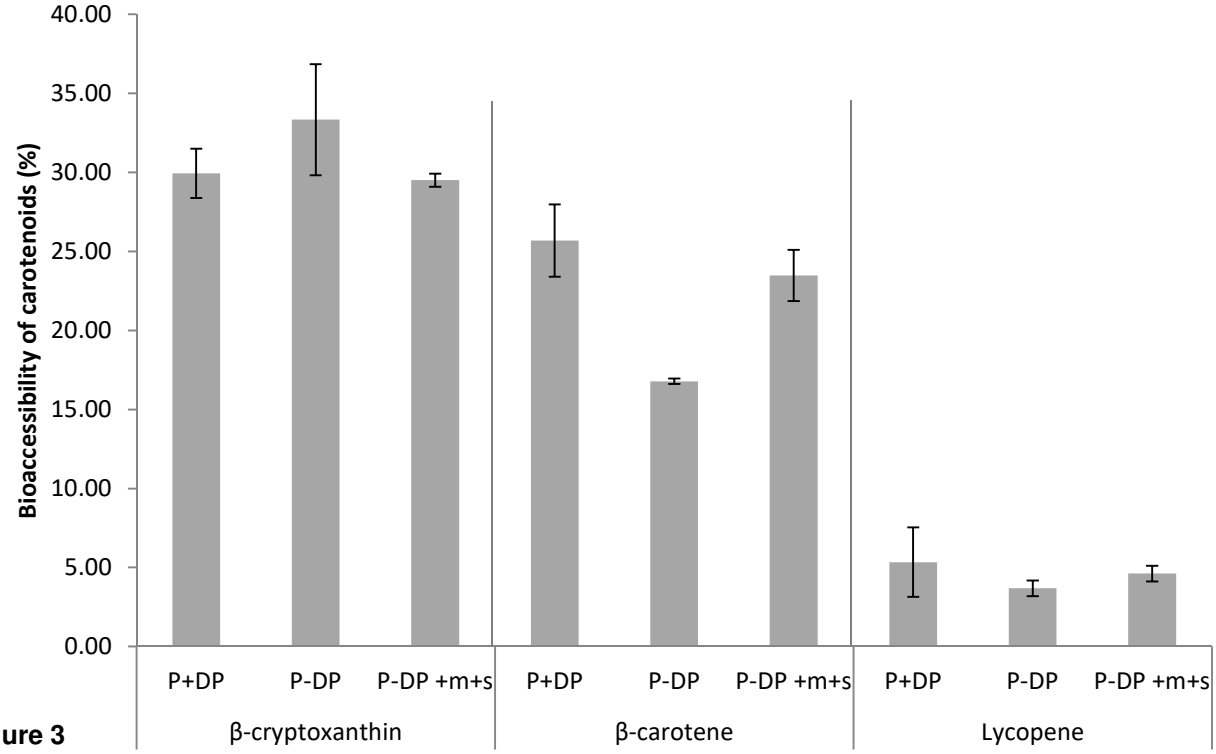
Bioaccessibility of carotenoids (%)
40.00
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
P+DP
P-DP
P-DP +m+s
P+DP
P-DP
P-DP +m+s
P+DP
P-DP
P-DP +m+s
β-cryptoxanthin
β-carotene
Lycopene

Figure 3

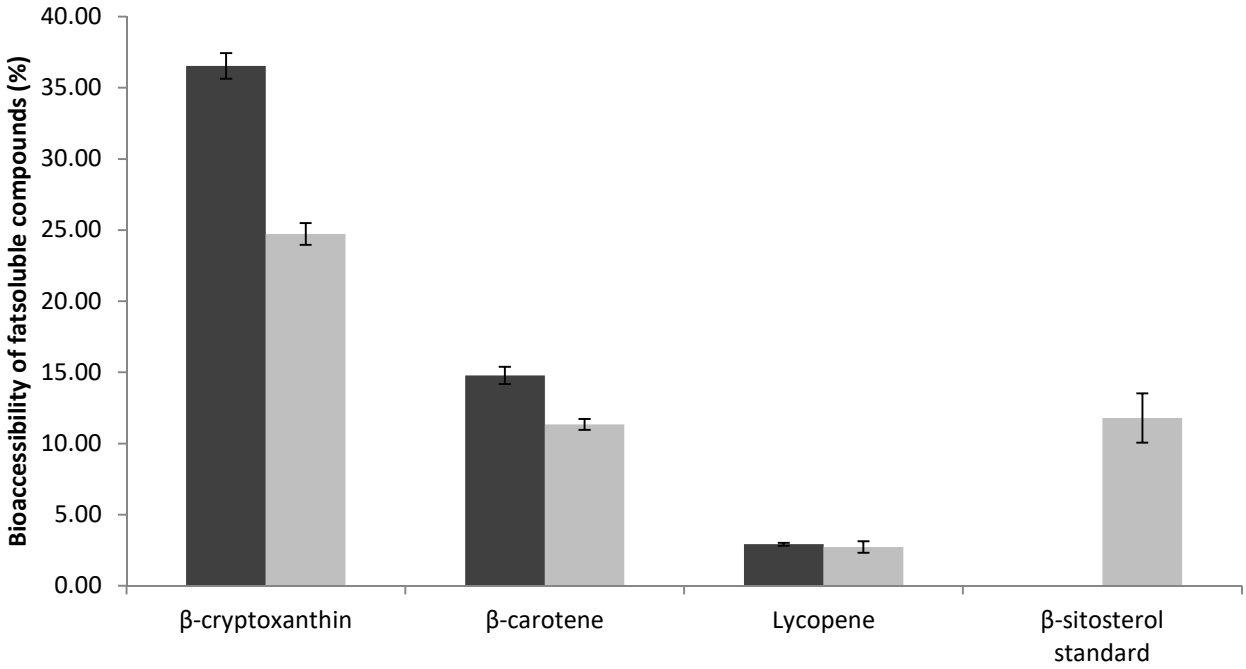

Figure 4

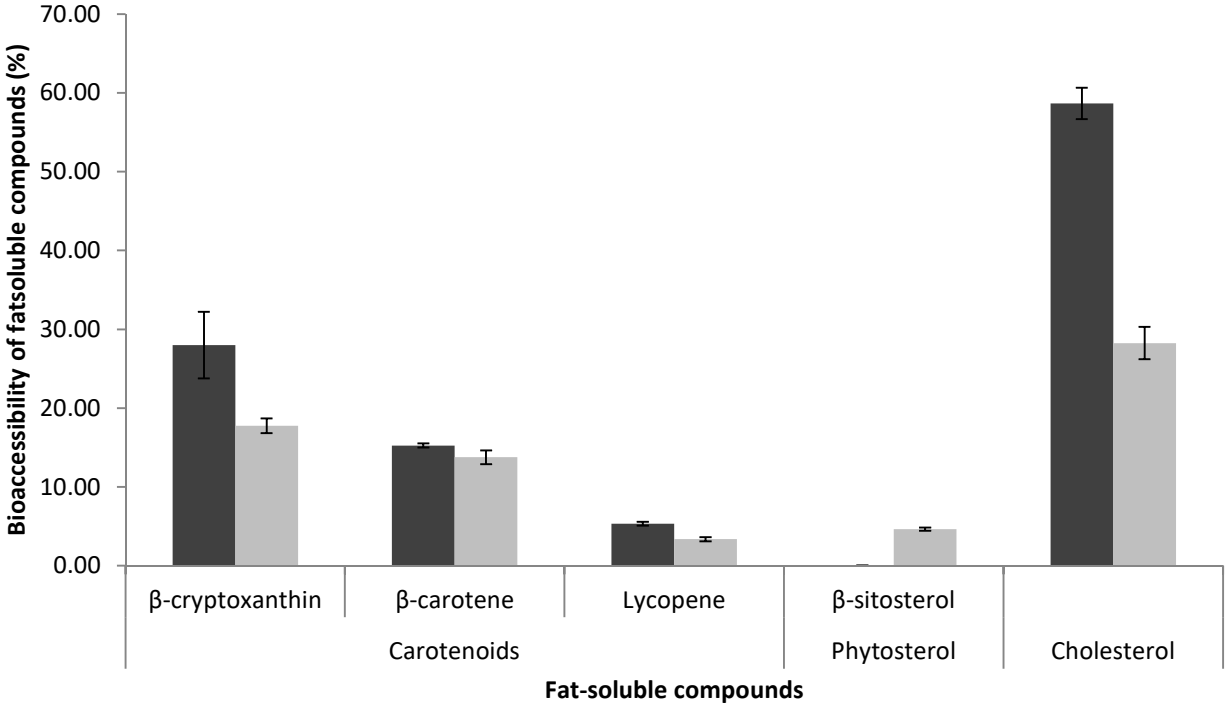

Table 1 : Fat-soluble compound contents of the fermented maize yogurt-like product

Phytochemicals	Content/kg	Content/serving portion
Carotenoids	**mg/kg**	**mg/125 g portion**
β-cryptoxanthin	1.90 ± 0.04	3.29 ± 0.10
β-carotene	14.55 ± 0.45	
Lycopene	9.85 ± 0.32	
Tocopherols	**mg/kg**	**mg/125 g portion**
α-tocopherol	1.53 ± 0.05	0.48 ± 0.04
γ-tocopherol	2.28 ± 0.06	
Phytosterol	**g/kg**	**g/125 g portion**
β-sitosterol	16.84 ± 0.46	2.11 ± 0.06

568 Values are means of 3 independent determinations ± standard deviation (SD)
569

570

571 **Table 2**: Cellular uptake of fat-soluble compounds by Caco-2 cells

% UPTAKE	Bcx	Bc	Lyc	β-sitosterol	Cholesterol
P	18.9 ± 2.3^{c}	14.1 ± 1.6^{de}	7.2 ± 1.6^{g}	7.9 ± 1.3^{g}	-
P without DP	28.4 ± 2.4^{a}	21.3 ± 0.7^{bc}	10.4 ± 1.8^{ef}	-	-
P + chol	18.5 ± 0.8^{cd}	18.2 ± 0.6^{cd}	9.21 ± 1.9^{fg}	5.8 ± 0.4^{h}	0
P without DP + chol	24.9 ± 0.2^{ab}	25.4 ± 1.5^{ab}	12.31 ± 1.8^{ef}	-	2.1 ± 0.4^{i}

572 Values are means of 3 independent determinations ± standard deviation (SD)
573 Means with the same superscript (a–b) do not differ significantly (Tukey test, *p*-value ≤ 0.05)
574

575

576 **Table 3**: Size of mixed micelles after the *in vitro* digestion of different products

	Size (mean radius, nm)	
	Mean	*SEM*
P	9.41^{b}	0.20
P without DP	8.33^{c}	0.20
P without DP + vehicles	10.61^{a}	0.24

577 P: Product; DP: Dispersible Phytosterols; vehicles: maltodextrin/sucroesters (10/3%)
578 Means with the same superscript (a–b) do not differ significantly (Tukey test, *p*-value ≤ 0.05)