African *Adansonia digitata* fruit pulp (baobab) modifies provitamin A carotenoid bioaccessibility from composite pearl millet porridges

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Abstract

Food-to-food fortification of staple cereal products using nutrient-dense plants shows promise to address multiple micronutrient deficiencies including vitamin A, iron and zinc in Sub-Saharan Africa. However, there is limited information on the potential interaction effects that such food-to-food fortified strategies may have on individual micronutrient bioavailability. The main objective of the current study was to investigate the impact of incorporating Adansonia digitata (baobab fruit pulp), a mineral-rich plant material, on the delivery of carotenoids from a composite cereal porridge. Formulations of native fruit/vegetable-cereal composites were screened for interactions which could influence both bioaccessibility and subsequent intestinal uptake of provitamin A carotenoids. Proportions of pearl millet flour and plant materials were dry blended to provide composite cereal porridges with total provitamin A carotenoid concentrations ranging from 3590.7±23.4 to 3698.5±26.5 μg/100g (fw) and baobab concentrations ranging from 0-25% (dw).While there were no significant differences in provitamin A carotenoid bioaccessibility from porridge formulations containing 5 or 15% baobab, inclusion of 25% baobab resulted in a significant ($P<0.05$) decrease in bioaccessibility (13.3%) as compared to the control (23.8%). Despite the reduced bioaccessibility, 6h uptake efficiency of provitamin A carotenoids by Caco-2 human intestinal cells was not significantly altered by 25% baobab inclusion. These findings suggest that the inhibitory effects on carotenoid micellarization (bioaccessibility) observed with increased baobab addition may not ultimately limit the bioavailability of carotenoids.

Keywords: Provitamin A carotenoids, Bioaccessibility, Baobab, Micronutrients, Fortification

Introduction

Deficiencies of vitamin A, iron and zinc, remain as the most prevalent public health challenges in Sub-Saharan Africa (Nair et al. 2016). An estimated 190 million preschool-aged children are affected by vitamin A deficiency of which Sub-Saharan Africa accounts for about 48% of all reported cases (WHO 2009). Strategies to address this nutritional challenge include supplementation, diet diversification and fortification through traditional or, more recently, biofortification routes. While many traditional diets in these regions are composed of staples including grains and starch rich foods (cassava and potato), these foods are typically low in shortfall micronutrients including iron, zinc and vitamin A (Chivandi et al. 2015). In this context, diet diversification and bio/fortification, as well as food-to-food fortification strategies that leverage local agricultural commodities are increasingly being considered, and in some cases implemented, as potential sustainable approaches that can simultaneously combat multiple micronutrient deficiencies (Nair et al. 2016).

Vitamin A derived from locally available fruits and vegetables in the form of provitamin A carotenoids substantially contribute to the vitamin A intake in Sub-Saharan Africa (WHO 2009). Carotenoids, a class of lipid-soluble plant pigments abundant in in dark green, orange and red plant foods, have been associated with many health benefits including reduced risk of several chronic and degenerative diseases (Milani et al. 2017). However, for the subclass of provitamin A carotenoids, vitamin A activity remains the most critical biological activity in humans. Among the 600+ carotenoids identified in nature, three main forms common in the human diet have provitamin A activity including β-carotene, α-carotene, and β-cryptoxanthin (Milani et al. 2017). Common sources of provitamin A carotenoids in the African diets include carrots, mangoes, papayas,

orange-fleshed sweet potato flesh and leaves, pumpkin leaves, squash and red palm oil (Uusiku et al. 2010).

While these fruits and vegetables have the potential to provide sufficient vitamin A as individual ingredients or as components of blended foods and diets that apply food-to-food fortification strategies, they are highly perishable and known to be subject to high post-harvest losses leading to a situation of limited year-round availability (Affognon et al. 2015). One possible solution is to develop non-perishable provitamin A rich ingredients from these materials and incorporate these ingredients into staple foods in a food-to-food fortification approach. However, to date, only limited information is available on their efficacy in the context of processed food products, such as extruded porridges, that are being consumed more frequently in sub-Saharan Africa. Similarly, indigenous plants being leveraged for minerals in this region include baobab, moringa, amaranth, cowpea, pumpkin seed, and spider flower (Uusiku et al. 2010). Baobab (*Adansonia digitata)* is of particular interest as it is not only rich in minerals but also in ascorbic acid which is known to enhance the bioavailability of iron (Kamatou et al. 2011). Baobab fruit pulp is commonly found in several parts of Sub-Saharan Africa where it is used for medicinal purposes as well as an ingredient for common beverage and food preparation (Kamatou et al. 2011). A recent study by Van der Merwe et al. (2019) demonstrated that the incremental addition of baobab fruit pulp in a pearl millet porridge co-formulated with provitamin A carotenoid rich carrot and mango resulted in an increase iron and zinc bioaccessibility as compared to other formulations containing mineral-rich native plant materials. While this finding shows promise for leveraging baobab fruit as an enhancer of mineral bioaccessibility, the impact on the bioavailability of carotenoids from these products is not known.

Carotenoid bioavailability depends on multiple factors including their food matrix, food preparation and processing methods as well as the presence of other nutrients or factors such as lipids, fibre and minerals that may impact the ability of carotenoids to be released from the food matrix during digestion and associate with lipid micelles for transport into the epithelia (Desmarchelier and Borel 2017). Corte-Real et al. (2016) demonstrated that bioaccessibility of pure carotenoids was significantly reduced with the addition of calcium, magnesium and zinc with calcium having the highest impact in limiting micellarization (up to 100% reduction). Consequently, while food-to-food strategies that promote the simultaneous incorporation of vitamin A and mineral rich food ingredients are of great interest, the impact of this complex mixture on the bioavailability of provitamin A carotenoids needs to be clarified.

The present study investigated the impact of incorporating baobab fruit pulp as a natural iron fortificant, on the bioaccessibility of provitamin A carotenoids from dried carrot and mango blend co-formulated in commonly consumed millet porridges. Considering previous reports showing the negative impact of high mineral content on the formation of mixed micelles thereby reducing the bioaccessibility of carotenoids, we hypothesized that the addition of mineral rich baobab would negatively impact the bioaccessibility of provitamin A carotenoids from a blended millet-fruit/vegetable thin porridge.

Materials and methods

Materials

Authentic carotenoid standards including β-carotene, β-cryptoxanthin, as well as β-apo-8′-carotenal (internal standard) were purchased from Sigma (St. Louis, MO, USA). α-cryptoxanthin, and α-carotene were from CaroteNature (Lupsingen, Switzerland). HPLC and ACS grade solvents

petroleum ether, acetone, hexane, ethyl acetate, and methanol were purchased from J. T. Baker (Phillipsburg, NJ, USA) and methyl tert-butyl ether was from Sigma-Aldrich (St. Louis, MO). Ammonium acetate, butylated hydroxytoluene (BHT) and potassium hydroxide were from Sigma-Aldrich (St. Louis, MO). In *vitro* digestive enzymes including α-amylase, porcine pepsin, pancreatin, lipase, and bile extract were obtained from Sigma-Aldrich. For cell culture experiments, Dulbecco's Modified Eagles Medium (DMEM), non-essential amino acids (NEAA), penicillin/streptomycin (pen/strep), and phosphate buffered saline (PBS) were purchased from Lonza (Walkersville, MD, USA). Fetal Bovine Serum was purchased from Atlanta Biologicals (Atlanta, GA). 1% v/v solution of 4-(2-hydroxylethyl)-1-piperazineethanes (HEPES) was prepared using double-distilled water, adjusted to pH 7.2 and autoclaved. Gentamycin were obtained from J.R. Scientific (Woodland, CA, USA), and trypsin from Thermo Scientific (Waltham, MA, USA). Bovine serum albumin (free fatty acid free) (FFA), sodium taurocholate and QuantiPro BCA Assay Kit were obtained from Sigma-Aldrich (St.Louis, MO, USA). Whole grain pearl millet (Senegalese Mil Souna var.) was received from Alif Group in Dakar, Senegal which was later decorticated and extruded at Purdue University's Department of Food Science (West Lafayette, IN. USA). Fresh carrots and mangoes were purchased from a local market. Dried baobab fruit pulp samples were received from Free Works Production (Dec. 2016, Dakar, Senegal).

Preparation of millet-based composite porridges:

Test materials for composite porridges consisted of traditional cereal (pearl millet), dried baobab fruit pulp and dried carrot and mango (provitamin A sources) which would be available seasonally and locally in Senegal. The amount of baobab and carrot/mango blend added in the formulation were designed to meet ~25% of the Recommended Daily Allowance (RDA) for iron and ~30% of the RDA for vitamin A respectively for children aged 1-3 yr.

Prior to test porridge preparation, the millet was decorticated and fully cooked by extrusion using a Technochem Mini-Extruder© (speed fixed at 900 rpm; final temperatures ranging between 105-121 °C). For the purpose of applying and translating the current work into the field, the low-cost extruder utilized was similar to the ones that have been deployed by Feed the Future Innovation Lab for Food Processing and Post-harvest Handling (FPL) in target regions of sub-Saharan Africa (https://ag.purdue.edu/ipia/fpl). The extruded millet powder was sieved (mesh size openings of 180 to 300 µm) to control for particle size differences. Carrots and ripe mangos were trimmed and sliced into 1mm diameter using Professional Grade Quality Kitchen mandoline, freeze-dried (Labconco Freezone 18 L, Kansas, MO, USA) and ground in a blender (Cuisinart Spice and Nut Grinder, East Windsor NJ, USA) to generate a powdered ingredient.

Preparation of final test porridges followed formulas described in Table 1. These products were designed to emulate thin porridges commonly consumed in Senegal and prepared as previously described with modifications (Lipkie et al. 2013). Briefly, individual ingredients were directly weighed into a falcon tube for a total of ~1.9 g of dry mix. The porridge slurry was then prepared by adding 8 ml of distilled boiling water (1:4, dry mix to water ratio), agitated on a vortex mixer for 5 min for thorough mixing, and placed under room temperature for 30 min before storage at -80C or further analysis or digestion.

Table 1. Formulation and carotenoid content of food-to-food fortified millet porridge meals[1,2,3]

	Control	Baobab at 5%	Baobab at 15%	Baobab at 25%
Ingredients (% wt./wt.)				
Water	80.0	80.0	80.0	80.0
Decorated & extruded Souna millet	13	12	10	8
Carrot & mango blend (1:1)	6	6	6	6
Baobab	0	1	3	5
Sunflower oil (5% of the dry mix)	1	1	1	1
Total wet porridge (dry mix + water) %	100	100	100	100
Carotenoid content (µg/100g fresh wt)				
β-cryp	18.3 ± 0.9	17.8 ± 0.8	17.1 ± 1.0	15.7 ± 0.6
All-trans-αC	1728.1 ± 13.3	1719.2 ± 27.8	1679.3 ± 4.6	1715.8 ± 19.3
All-trans-βC	2719.5 ± 23.3	2707.8 ± 40.5	2635.9 ± 22.5	2694.5 ± 34.4
cis-βC	211.6 ± 7.0	200.9 ± 10.8	213.2 ± 5.4	219.3 ± 7.0
Total Provitamin A carotenoids (TPVA)	3698.5 ± 26.5	3676.7 ± 58.2	3590.7 ± 23.4	3669.9 ± 45.3

[1]Reported values represent mean ± SEM (standard error of the mean) (n=4). [2]Abbreviations used: β-cryptoxanthin (β-CRP), all-*trans*-α-carotene (all-trans-αC), all-*trans*-β-carotene (all-trans-βC), *cis*-β-carotene isomers (cis-βC). [3]TPVA represents total provitamin A carotenoids calculated as β-carotene equivalent: all-trans-βC + 1/2(β-cryp + all-trans-αC + cis-βC). No significant differences ($p > 0.05$) as analysed by Tukey's test were observed between porridge meals within individual carotenoid species.

Mineral, total phenolics and proximate analysis of dried baobab fruit pulp

Dried baobab fruit pulp (~ 0.5 g) was dissolved in 70% HNO_3 and digested in a closed-vessel microwave oven. Following digestion and subsequent cooling, ashed baobab powder was diluted with double-distilled water to a final concentration of 2% NHO_3 prior to mineral analysis. Analysis of calcium, magnesium, sodium, iron and zinc was carried out using inductively coupled plasma-optical emission spectrometry (ICP-OES; Optima 4300DV, Perkin Elmer, Shelton, CT, USA). For total phenolic content, 0.05 g of dried baobab pulp (n =3) was first defatted using hexane and dried under nitrogen. The defatted sample was then subjected to phenolic extraction using 80% methanol containing 1% formic acid three times. The supernatant was dried and resolubilized with 0.1% formic acid prior Folin-Ciocalteu assay (Sánchez-Rangel et al. 2013). Total phenols in baobab fruit

pulp was quantified as gallic acid equivalents (GAE, mg/100g) after adjusting for vitamin C and other reducing compounds (Sánchez-Rangel et al. 2013). Proximate analysis including crude protein (nitrogen x 6.25), nitrogen (AOAC 968.06), crude fat (AACC 30-20), ash (AOAC 942.05), crude fibre (AACC 32-10), and carbohydrates (100 – (crude fat + Ash + crude fibre + moisture)) for dried baobab fruit pulp was conducted by A&L Great Lakes Laboratories (Fort Wayne, IN).

Carotenoid bioaccessibility from blended cereal porridge samples

Prior to digestions, porridges were thawed and brought to room temperature. To facilitate the incorporation of carotenoids into mixed micelles, ~0.1 g (1% w/w) sunflower oil was added to the 9.9 g of prepared porridge for a total of 10 g prior to initiation of the *in vitro* digestion experiment. Each 10 g porridge sample was then subjected to a three-stage simulated digestion consisting of an oral, gastric and small intestinal phase as previously described with minor modifications (Lipkie et al. 2013). Briefly, the oral phase solution (6 mL) containing α-amylase (3000 units) was added into the porridge sample. The reaction tube was then thoroughly mixed on a vortex for 1 min, flushed with nitrogen and incubated for 10 min in a shaking incubator at 120 rpm. Subsequently, samples were subjected to a gastric phase which consisted of the addition of 2 mL pepsin solution in 0.1 M HCl (10 mg/mL) and pH adjustment to 2.5 using 1 M HCl. The reaction tube volume was then brought to 40 mL with 0.9% saline solution, flushed with nitrogen and incubated (120 rpm) at 37 °C for 1hr. The intestinal phase was initiated by adding 2 mL solution of pancreatin (20 mg/mL) and lipase (10 mg/mL) along with 3 mL bile extract (30 mg/mL) in 0.1 M $NaHCO_3$. The pH of the digesta mixture was then adjusted to 6.5 with 1 M $NaHCO_3$ and incubated at 37 °C for 2hr. Following the intestinal digestion, aliquots of chyme were collected and stored at -80C while a separate aliquot of the same digesta sample was centrifuged at 10,000g at 4 °C for 1 h to isolate carotenoids sequestered in mixed micelles within the aqueous fraction from solid residues and

other insoluble particles. The aqueous fraction was then filtered with 0.22 μm Polytetrafluoroethylene (PTFE) membrane filter to isolate the mixed micelle fraction which was collected and stored at -80 °C until further analysis.

Caco-2 cell culture and cellular uptake experiment

Following *in vitro* digestion, carotenoid uptake from aqueous digesta fractions by the human intestinal cells was evaluated using differentiated human intestinal Caco-2 cells (HTB-37 parental cell line, American Type Culture Collection). Cells were maintained as previously described with minor modifications (Ferruzzi et al. 2006). Caco-2 cells were grown at 37 °C with 5% CO_2 atmosphere and passaged at ~80-85% confluency. Cells were maintained using complete media composed of DMEM containing 10% v/v FBS, 1% v/v NEAA, 1% v/v HEPES, 1% v/v pen/strep and 0.1% v/v gentamicin where the media was changed every 2 days and 24 hours before uptake experiment. Cells at passages 23-29 were then grown on 6-well plates at a seeding density of 1.28 x 10^5 cells/well and were used for uptake experiments 10-12 days post confluency. To evaluate carotenoid uptake and accumulation by the Caco-2 cells, the differentiated monolayers were rinsed with 0.1% fatty acid free albumin in PBS at 37 °C followed by PBS at 37 °C. The monolayers were then incubated with test media containing (1:4) aqueous digesta and DMEM for 0, 2, 4 or 6 hr. Following treatment, monolayers were washed with 5 mM sodium taurocholate in PBS at 37 °C post incubation to remove residues of mixed micelles and surface carotenoids and harvested by scraping in ice cold PBS (Ferruzzi et al. 2006). Protein content of treatment wells were evaluated using QuantiPro BCA Assay. Cytotoxicity of treatments was evaluated using MTT assay.

A second experiment was also conducted to isolate the potential effects of baobab fruit pulp alone on carotenoid intestinal uptake without a digested porridge background matrix. For this

experiment, 0.5 g of baobab (to match baobab content of 25% baobab porridge sample), 1 g of fruit blend (provitamin A rich carrot: mango, 1:1) and 0.9% saline sample (blank) were separately subjected to *in vitro* digestion and aqueous fractions from each were blended in a ratio of 1:0:1 (control), 1:1:1 (baobab_1x) or 1:2:0 (baobab_2x) respectively for the preparation of treatment media. Each of these combinations of aqueous fraction was then diluted with serum-free DMEM in a ratio of 1:3. Caco-2 monolayers were incubated with treatment media for 0, 3 and 6hr. Collected cells were stored under nitrogen at – 80 °C until analysis.

Carotenoid extraction and analysis

Carotenoid analysis from porridge samples as well as fractions collected from the digestion process were carried out according to the procedure as previously described (Lipkie et al. 2013). Briefly, prepared porridge samples (0.5 g) were extracted with acetone followed by methyl tert-butyl ether (MTBE) containing 0.1% butylated hydroxytoluene (BHT). Digested porridge fractions including the intestinal digesta and aqueous micellar fractions were extracted using 0.1% BHT acetone: petroleum ether (1:3). Carotenoids from harvested cells were extracted in similar fashion as digested fractions once cell pellets were homogenized with a Branson 450 for 5 s at 20 watts. Carotenoids were analysed using Waters ACQUITY UPLC equipped with diode array detector. Separation of carotenoids was carried out using YMC C30 3µm, 2.0 mm × 150 mm column with a gradient of ethyl acetate and methanol and 2mM ammonium acetate as previously described (Kean et al. 2008). Identification and quantification were carried out using Waters Masslynx software at 450 nm based on calibration curves of authentic standards for all-*trans*-carotenoids whereas *cis*-isomers were identified using previously reported UV-Vis absorption

spectra and retention times and quantified based on all-*trans* response as previously reported (Kean et al. 2008).

Data analysis

Data for carotenoid content of porridge samples, digesta and aqueous fraction as well as intracellular content are expressed as mean ± SEM for a minimum three replicates for each experiment. Micellarization efficiency (relative bioaccessibility) is expressed as the ratio of the carotenoid content in the aqueous fraction to that of the digesta fraction of the porridge samples. Bioaccessible content (µg/100g, FW) is defined as the fraction of carotenoids in the starting material (porridge, µg/100g, FW) that is available for absorption as derived from the micellarization efficiency. Total provitamin A carotenoid content (TPVA) was calculated as the sum of all-*trans*-β-carotene + 1/2(α-cryptoxanthin + β-cryptoxanthin + α-carotene + *cis*-β-carotene). The conversion factor of 1 µg RAE = 12 µg of β-carotene was used to estimate the RDA levels that could be met with a serving of each porridge formulation (Grune et al. 2010). Carotenoid uptake efficiency (%) by Caco-2 cells was expressed as the ratio of Caco-2 carotenoid intracellular content to that of the aqueous fraction content within treatment media. Significant differences ($p < 0.05$) in individual carotenoid species and TPVA content between porridge formulations, micellarization efficiency data, bioaccessible content, cellular carotenoid content and cellular uptake efficiency were compared by analysis of variance (ANOVA) using JMP version 12 (SAS Institute, Cary, NC) with Tukey-Kramer Honestly Significant Difference (HSD) method post-hoc test.

Results and discussion

Provitamin A carotenoid composition of porridge meals:

The major provitamin A carotenoids in the porridge test meals were all-*trans*-β-carotene, α-carotene, and β-cryptoxanthin consistent with previous reports of fresh and processed carrots and mangoes (Bozalan and Karadeniz 2011; Burton-Freeman et al. 2017). Isomers of β-carotene including 15-*cis*-β-carotene, 13-*cis*-β-carotene and 9-*cis*-β-carotene were also detected and presumably derived from the drying/preparation process as well as some natural levels of *cis* isomers present in carrot and/or mango (Hiranvarachat et al. 2008). Total provitamin A carotenoid content of prepared porridge meals ranged from 3,590.7 ± 23.4 to 3,698.5 ± 26.5 μg/100g (fresh weight), with no significant differences between the porridge formulations ($p > 0.05$) (Table 1). Consistent with previous reports on carrots and mango, all-*trans*-β-carotene was the predominant provitamin A carotenoid contributing ~74% of the total provitamin A carotenoid concentration (Ma et al. 2015). α-carotene contributed ~23%, taking into account that α-carotene has been reported to have ~50% the provitamin A activity as β-carotene (Grune et al. 2010). β-cryptoxanthin concentration was the lowest among all the carotenoid species identified contributing only ~0.24% of the total provitamin A carotenoid concentration. Total *cis*-isomers accounted for only ~2.9% of total provitamin A carotenoids which is consistent with previous reports showing the presence of β-carotene isomers in fresh and processed carrots (Knockaert et al. 2012).

Bioaccessibility of provitamin A carotenoids from porridge meals:

The main objective of this study was to determine the potential effects of food-to-food fortification of millet porridges with natural sources of provitamin A (carrot and mango) and

mineral (baobab) on the bioaccessibility of provitamin A carotenoids. Using a three-stage in *vitro* digestion model, micerallization efficiency (relatively bioaccessibility, %) from formulations (matched for provitamin A content) with increasing proportions of baobab fruit were assessed. Overall, micellarization efficiency was found to be negatively impacted by the presence of baobab in a dose-dependent fashion (Figure 1). Micellarization of TPVA ranged from 13.4% (baobab at 25%), 18.58% (baobab at 15%), 20.46% (baobab at 5%), to 23.5 ± 1 (control). While there were no significant differences in total provitamin A carotenoid micellarization with lower baobab addition (baobab at 5 and 15%) into porridge formulations as compared to the control (baobab at 0%), the presence of baobab at 25% significantly reduced ($p < 0.05$) total provitamin A carotenoid bioaccessibility.

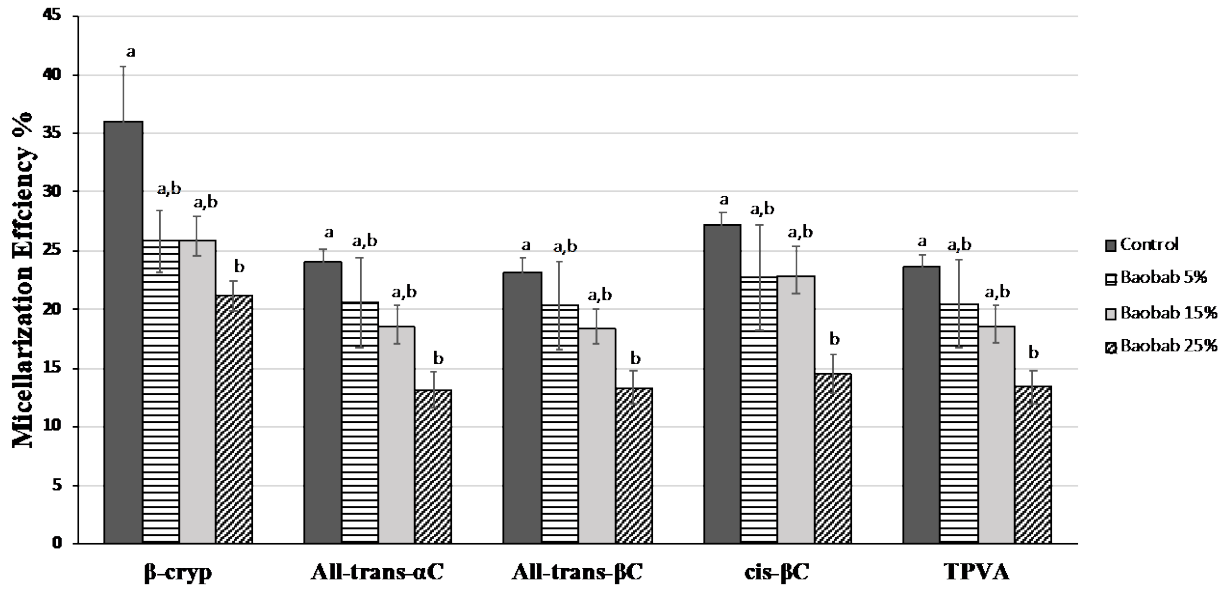

Figure 1. Percent micellarization efficiency (relative bioaccessibility) of individual carotenoid species from control, 5%, 15%, and 25% baobab food-to-food fortified porridge meals following *in vitro* digestion. All data presented are expressed as mean ± standard error (n=4). Different letters indicated above the bars represent significant differences in micellarization efficiency (%) between porridge formulations within individual carotenoid species ($p < 0.05$). Abbreviations used: β-cryptoxanthin (β-CRP), all-*trans*-α-carotene (all-trans-αC), all-*trans*-β-carotene (all-trans-βC), *cis*-β-carotene isomers (cis-βC), TPVA (total provitamin A carotenoids).

Reduction of micellarization efficiency may be due to several factors including the contribution of divalent minerals from baobab fruit pulp that may interfere with the formation of mixed micelles. Minerals analysis of the baobab utilized in this study was found to contain high levels of calcium (292.3 mg/100 g) and magnesium (and 150.1 mg/100 g) (Table 2). This finding is in agreement with the study by van der Merwe et al. (2019) who reported calcium of baobab fruit pulp collected from similar production location to be 273 mg/100 g. A study by Corte-Real et al. (2017) showed that the micellarization efficiency of β-carotene from carrots was significantly reduced with increased levels of calcium (0 -1000 mg/l) and magnesium (0 – 300 mg/L) (Corte-Real et al. 2017). The authors demonstrated that increased levels of divalent minerals resulted in higher surface tension and macro-viscosity indicating the loss of surfactants (bile salts) from the micellar structure. The divalent metals may also cause precipitation of insoluble fatty acid soaps as well as bile salts by binding to unconjugated fatty acids and bile acids (Biehler et al. 2011b; Corte-Real et al. 2016, 2017). These factors are likely lead to disruption of mixed micelle formation and/or stability resulting in and overall reduction of carotenoid bioaccessibility.

Table 2. Proximate composition, mineral and total phenolic content of dried baobab fruit pulp[1]

Proximate analysis (g/100g)					
Moisture	**Crude Protein**	**Crude Fiber**	**Crude Fat**	**Ash**	**Carbohydrates**
7.1± 0.1	2.5± 0.1	11.2± 0.4	0.5± 0.0	4.5± 0.0	74.3± 0.4

Mineral content (mg/100g)						Total phenols (mg/100g)
Calcium	**Magnesium**	**Sodium**	**Phosphorus**	**Iron**	**Zinc**	**Gallic acid eq.**
292.3 ± 22.9	150.1 ± 10.5	trace	50 ± 6.9	11.8 ± 2	trace	3360.5 ± 247.4

[1]Reported values represent mean ± standard error (n=3). Total phenols in baobab fruit pulp is expressed as gallic acid equivalents (GAE).

Additional potential factors limiting the micellarization of carotenoids could be the presence of specific phenolics (flavan-3-ols) and polysaccharides (xyloglucans) in baobab fruit as characterized by our group (Debelo et al. in review). The work by Van der Merwe et al. (2019) demonstrated that flavonoids consisting of the subclasses, flavan-3-ols and flavonols were the predominant phenolic compounds found in baobab fruit pulp obtained from similar production centre as the present study. Flavan-3-ols, particularly procyanidins have been shown to limit pancreatic lipase activity in mice and in human known to contribute to their role in management of obesity and diabetes (Sugiyama et al. 2007). As flavan-3-ols have been shown to be major constituents of phenolics in baobab fruit pulp (Braca et al. 2018; van der Merwe et al. 2019), the limited micellarization of carotenoids may be partially attributed to this inhibitory effect of lipase activity by these classes of compounds. In terms of polysaccharides, while the effect xyloglucan (identified as soluble fraction of fibre) on the bioaccessibility and bioavailability of carotenoids has not been investigated, other soluble and insoluble fibre have been shown to play a role in carotenoid micellarization by precipitating lipid digestion biproducts and bile salts (Tomas et al. 2018). It could be expected that xyloglucans from baobab fruit might have similar inhibitory effects although further *in vitro* and *in vivo* studies are required demonstrating the binding effect of xyloglucan isolated from baobab on bile salts and other micellar components to establish their direct effect on carotenoid bioavailability.

Similar negative effects were observed on the micellarization efficiency of individual carotenoid species with the addition of 25% baobab (Figure 1). All carotenoid species had a significantly lower micellarization ($p < 0.05$) in porridge containing 25% baobab compared to control. However, significance of this effect was not observed at lower levels of 15 and 5% suggesting that lower levels of baobab fruit pulp can be used without significant effect on

provitamin A bioaccessibility. This is critical as Van der Merwe et al. (2019) reported that the addition of baobab even at ~10% in similar porridge formulations could potentially provide ~20-25% (iron) and ~35-40% (zinc) of the absolute requirement for women of reproductive age suggesting a combined approach is possible.

Interestingly, β-carotene *cis* isomers demonstrated higher micellarization than all-*trans* isomer, consistent with previous observations on the micellarization efficiency of cis-isomers using a similar model (Ferruzzi et al. 2006; Failla et al. 2008). However, despite the higher relative bioaccessibility of the *cis* isomers, all-*trans*-β-carotene was the major carotenoid present in the bioaccessible fraction contributing ~72% of the total provitamin A carotenoids followed by α-carotene (~24%).

Bioaccessible carotenoid content indicates the amount of carotenoids available from a serving of raw material adjusted by the micellarization efficiency (raw material content x % micerallization efficiency). The bioaccessible content of the porridge samples from a modest but reasonable serving size (200 g fresh weight) is presented in Table 3. Despite the similarities in TPVA content of all undigested porridge meals, the bioaccessible content was significantly lower ($p < 0.05$) from the formulation containing baobab at 25% (982.6 µg per serving) than that of the control (1743.4 µg per serving). However, the bioaccessible content of baobab at 5% (1506 µg per serving) or 15% (1334.5 µg per serving) was not significantly different from the control. While porridge formulation containing baobab at 25% had significantly lower amounts bioaccessible α-carotene, β-carotene and cis isomers than that of the control, there was no significant difference between control and baobab at 5 and 15% for these carotenoid species. However, the bioaccessible content of β-cryptoxanthin was significantly higher from the control porridge (13.2 µg per serving) than all the other formulations (6.6 – 9.1 µg per serving). Overall, considering the high correlation of

the mean carotenoid bioavailability from a similar *in vitro* digestion model to that of healthy human studies (Reboul et al. 2006), the contribution of these porridge formulations to the RDA of vitamin A can be estimated. The consumption of 200 g porridge meals containing 0 (control), 5, 15 or 25% of baobab has the potential to meet 48%, 41%, 37% or 27% of the RDA of vitamin A for children 1-3 years of age. These data indicate that the consumption of these porridge meals could substantially contribute to vitamin A requirements despite the reduced bioaccessible content of provitamin A carotenoids with increased amounts of mineral-rich baobab fruit pulp.

Table 3. Absolute bioaccessible content of provitamin A carotenoids from porridge meals (µg/per 200 g of serving)[1,2,3]

	Porridge Formulations			
Carotenoids	**Control**	**Baobab 5%**	**Baobab 15%**	**Baobab 25%**
β-cryp	13.2 ± 1.6a	9.1 ± 0.6b	8.9 ± 0.8b	6.6 ± 0.2b
All-trans-αC	830.6 ± 32.9a	707.7 ± 128.5ab	623.3 ± 56.5ab	453 ± 50.5b
All-trans-βC	1263.7 ± 52.7a	1102.1 ± 206.7ab	970.2 ± 86.6ab	720.8 ± 78.9b
cis-βC	115.6 ± 7.4a	90.6 ± 16.1ab	98.2 ± 12.2ab	63.4 ± 6.1b
TPVA	1743.4 ± 72.6a	1506 ± 279.1ab	1334.5 ± 120.7ab	982.6 ± 106.7b

[1]Data shown represent absolute bioaccessible content calculated as the (raw material carotenoid content, µg/200g fresh weight) x (micellarization efficiency, %).
[2]Different letters represent significant differences in absolute bioaccessibility between porridge formulations within individual carotenoid species ($p < 0.05$). Values are mean ± standard error of the mean of n=4 analysis.
[3]Abbreviations used: β-cryptoxanthin (β-CRP), all-*trans*-α-carotene (all-trans-αC), all-*trans*-β-carotene (all-trans-βC), *cis*-β-carotene isomers (cis-βC), TPVA (total provitamin A carotenoids).

Provitamin A carotenoid uptake and accumulation efficiency

To confirm availability of bioaccessible carotenoids, carotenoid uptake/accumulation by human intestinal cells was assessed using differentiated Caco-2 human intestinal cells. Prior to the Caco-2 uptake experiment, treatment media was prepared using micellar fractions of digested

porridge samples diluted with DMEM (1:4). Caco-2 cells were then incubated with treatment media from 0-6h. Among the provitamin A carotenoids detected in the micellar fraction, all-*trans*-β-carotene and α-carotene were the only provitamin A carotenoids identified in harvested Caco-2 monolayers. Treatment media from control (568.8 ± 18.7 pmol/well) and baobab at 5% (515.3 ± 41.7 pmol/well) porridge meals had significantly greater ($p < 0.05$) all-*trans*-β-carotene content than baobab at 15% (347.2 ± 2 pmol/well) and 25% (198.2 ± 5.9 pmol/well). Similarly, α-carotene concentration was significantly greater in control (377.0 ± 13.8 pmol/well) and baobab at 5% (335.2 ± 27.5 pmol/well) than in baobab at 15% and 25% (222.6 ± 17.9 and 123.5 ± 3.5 pmol/well respectively). As reflected in the treatment media, significant differences in intracellular content of α- and β-carotene were observed (Table 4). At 4h incubation period, α-carotene accumulation was significantly lower in Caco-2 cells treated with diluted aqueous fraction containing baobab at 15% (4.5 ± 0.7 pmol/mg of protein) and baobab at 25%(3.1 ± 0.8 pmol/mg of protein) as compared to the control (8.0 ± 0.8 pmol/mg of protein). At 6h, intracellular content of α-carotene was only significantly lower in cells treated with baobab at 25% (4.4 ± 0.6 pmol/mg of protein) as compared to the control (12.3 ± 0.8 pmol/mg of protein). Similarly, intracellular content of β-carotene was significantly lower in cells treated with baobab at 25% (4h: 6.4 ± 1.5, 6h: 8.7 ± 0.7 pmol/mg of protein) as compared to the control (4h: 14 ± 1.6, 6h: 21.1 ± 4.4 pmol/mg of protein).

Table 4. Intracellular Caco-2 content of (pmol/mg of protein) and uptake efficiency (%) in parenthesis of α- and β-carotene after 0, 2, 4 and 6h of incubation with diluted micellar aqueous fraction from porridge meals containing 0, 5, 15 or 25% baobab[1,2,3]

	All-trans-αC				all-trans-βC			
Time	**control**	**Baobab 5%**	**Baobab 15%**	**Baobab 25%**	**control**	**Baobab 5%**	**Baobab 15%**	**Baobab 25%**
0hr	0 ± 0c (0 ± 0%)c	0 ± 0c (0 ± 0%)c	0 ± 0c (0 ± 0%)d	0 ± 0c (0 ± 0%)c	1.2 ± 0.3c (0.2 ± 0.1%)c	1.1 ± 0.2c (0.2 ± 0.03%)c	0.7 ± 0.2c (0.2 ± 0.1%)c	0.5 ± 0.2c (0.1 ± 0.2%)b
2hr	4.5 ± 1.4bc (1.2 ± 0.4%)bc	3.0 ± 0.9bc (0.9 ± 0.2%)bc	2.1 ± 0.7bc (0.9 ± 0.3%)c	1.6 ± 0.7bc (1.3 ± 0.6%)bc	7.3 ± 1.8bc (1.3 ± 0.3%)bc	5.8 ± 1.4bc (1.1 ± 0.3%)bc	4.5 ± 1.1bc (1.3 ± 0.3%)c	3.9 ± 1.1bc (2.0 ± 0.6%)ab
4hr	8.0 ± 0.8ab (2.2 ± 0.3%)ab	6.3 ± 0.8b (1.9 ± 0.3%)b	4.5 ± 0.7b* (2.0 ± 0.3%)b	3.1 ± 0.8ab* (2.6 ± 0.7%)ab	14.0 ± 1.6ab (2.5 ± 0.4%)ab	11.1 ± 1.5b (2.2 ± 0.4%)b	8.4 ± 1.4b (2.4 ± 0.3%)b	6.4 ± 1.5ab* (3.3 ± 0.8%)a
6hr	12.3 ± 2.5a (3.3 ± 0.7%)a	10.9 ± 1.6a (3.3 ± 0.5%)a	7.3 ± 0.8a (3.3 ± 0.2%)a	4.4 ± 0.6a* (3.6 ± 0.5%)a	21.1 ± 4.4a (3.7 ± 0.8%)a	19.1 ± 3.0a (3.7 ± 0.6%)a	13.2 ± 1.5a (3.8 ± 0.3%)a	8.7 ± 1.2a* (4.5 ± 0.7%)a

[1]The numbers in parentheses represent uptake efficiency (percent ratio of total cellular carotenoid content to that of the aqueous fraction, n=4 wells at each time point). Values are indicated as mean ± SEM.
[2]Asterisk (*) indicates significant differences ($p < 0.05$) in intracellular content or uptake efficiency (%), of treatment groups as compared to the control within each time point.
[3]Different letters indicate significant differences in intracellular content or uptake efficiency (%) between time points (0, 2, 4 or 6h) within each treatment group.

Despite the differences in intracellular content, there were no significant differences ($p > 0.05$) in the α- and β-carotene uptake efficiency of Caco-2 cells (percent ratio of intracellular carotenoid content to that of the treatment media) between the different treatment groups within each time point. By the end of the final incubation period (6h), β-carotene accumulation efficiency by Caco-2 cells ranged from 3.7 ± 0.8% (control), 3.7 ± 0.6% (baobab at 5%), 3.8 ± 0.3% (baobab at 15%) to 4.5 ± 0.7% (baobab at 25%). Similar trends were also observed for α-carotene where accumulation efficiency ranged from 3.3 ± 0.7% (control) to 3.6 ± 0.5% (baobab at 25%). The lack of significant differences in α- and β-carotene uptake efficiency by Caco-2 cells treated with different porridge formulations containing varying levels of baobab suggests that baobab fruit pulp has minimal impact of the active and/or passive transport of these carotenoids. However, the effect

of baobab alone on carotenoid uptake by Caco-2 cells was difficult to differentiate in this system due to the differences in α- and β-carotene content of the treatment media.

To further characterize any potential effect of baobab fruit pulp on carotenoid absorption by Caco-2 cells and to match the carotenoid content of treatment media, a second experiment was conducted using an isolated model. Briefly, micellar fractions of digested fruit blend (carrot: mango, 1:1) were incubated with separately digested baobab (0.5 g to match the content of baobab at 25% in porridge meals) for 0, 3 and 6h. To match the carotenoid content of all treatment samples, a blank digested sample (saline, 0.9%) was introduced and the treatments were prepared in ratios as described in the materials and methods section. The treatment combinations were then diluted with DMEM (1:3) to ensure detectable amounts of carotenoids was supplied to the cells for absorption. α- and β-carotene accumulation increased linearly with increased incubation time. Cellular content of α- and β-carotene ranged from 0 ± 0 (0h) - 5.97 ± 1.21 (6h) and 1.29 ± 0.26 - 11.84 ± 1.81 (6h) pmol/mg of protein respectively. There were no significant differences in α- and β-carotene accumulation between treatment groups at each time point.

The uptake efficiency of Caco-2 cells incubated with different treatment media over 0, 3 and 6h is presented in Table 5. By the end of the incubation period (6 hr), there were no significant differences in accumulation efficiency of both α-carotene (7.4 ± 1.4 - 7.8 ± 0.3%) or β-carotene (9.6 ± 1.3 – 10.7 ± 0.4%) between the different treatment groups. The uptake efficiency from the current study is consistent with a previous report by O'Sullivan et al. (2007) who investigated the absorption and transport of α- and β-carotene at varying levels using Caco-2 cells and demonstrated that the uptake efficiency ranged from 4.5% (0.5 μg) to 4% (2.5 μg) for β-carotene and 6.4% (0.5 μg) to 3.9% (2.5 μg) for α-carotene. In contrast, other studies have reported higher levels of uptake efficiency of these carotenoids ranging from 20-40% (Garrett et al. 2000; Ferruzzi

et al. 2001). These differences may be due to differences in dosage, form of carotenoid source (free vs. complexed in different food matrices), or differences in experimental conditions for Caco-2 cell culture. Overall, the current findings are in agreement with the *in vitro* studies mentioned above and previous clinical studies demonstrating similar absorption efficiency. A study by Hickenbottom et al. (2002) showed that the absorption of β-carotene ranged from 2 - 4% in humans as determined by a double-tracer study design. Similarly, α- and β-carotene absorption from processed carrots was found to be 1.2 – 3.5% and 0.9 – 2.4% respectively as determined by extrinsic stable isotope method which validate the use of Caco-2 cell models to predict carotenoid absorption by the enterocytes of the small intestine (Edwards et al. 2002).

Table 5. Uptake efficiency (%) of Caco-2 cells exposed to treatment media containing control or aqueous fraction of digested baobab sample at different levels incubated for 0, 3 or 6 h[1,2]

	All-trans-αC			all-trans-βC		
Time	**control**	**Baobab_1x**	**Baobab_2x**	**control**	**Baobab_1x**	**Baobab_2x**
0hr	0 ± 0c	0 ± 0c	0 ± 0c	1.0 ± 0.3d	1.3 ± 0.1d	1.1 ± 0.1d
3hr	3.4 ± 0.2b	4.7 ± 0.3b	4.6 ± 0.4b	5.1 ± 0.3c	7.0 ± 0.4c	7.3 ± 0.6bc
6hr	7.4 ± 1.4a	7.8 ± 0.3ab	7.5 ± 0.4ab	9.6 ± 1.3a	10.7 ± 0.4ab	10.6 ± 0.4a

[1]The description for the preparation of treatments is provided in the materials and methods section. [2]Each data point represents mean ± standard error of percent ratio of total cellular carotenoid content to that of the aqueous fraction (n=4 wells at each time point).
[3]Different letters indicate significant differences in Caco-2 cell uptake efficiency throughout the incubation period amongst treatment groups within each carotenoid species ($p < 0.05$).

Conclusion

Findings from the current study indicate that increased addition of baobab fruit pulp in porridge formulations containing provitamin A carotenoid-rich fruit blends seemed to have an inverse relationship with carotenoid incorporation into mixed micelles prior to their absorption by

the small intestine. While it is likely that the presence of high concentrations of divalent minerals in baobab fruit may cause the decrease in micellarization efficiency as previously demonstrated (Biehler et al. 2011a; Corte-Real et al. 2016), further research is warranted investigating the impact of baobab on the physiochemical parameters of micelles formed during the digestion process in order to establish direct relationship. However, despite the reduced bioaccessibility observed with increased amount of baobab, the absorption of target provitamin A carotenoids by Caco-2 human intestinal cells was not significantly altered in an acute setting. It remains to be seen if long-term exposure to baobab, as would be expected form introduction into staple foods, may alter lipid/carotenoid absorption by modulating expression of genes involved in carotenoid transport. It is estimated that porridge formulations following these food-to-food fortification strategies leveraging baobab fruit pulp (5-25% of dry weight) and provitamin A rich mango and carrot ingredients have the potential to meet 27-48% of the RDA of vitamin A for children 1-3 years of age. Along with ongoing consumer acceptability work in Senegal (Groote et al. 2018), the present study provides useful insights for translation of food-to-food fortification strategies containing native nutrient dense plant materials designed for the optimal delivery of carotenoids.

Conflict of interest and funding

The authors declare no conflicts of interest.

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