Food & Function

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Journal:	*Food & Function*
Manuscript ID	FO-ART-01-2022-000051.R1
Article Type:	Paper
Date Submitted by the Author:	01-Mar-2022
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Bioaccessibility and intestinal cell uptake of carotenoids and chlorophylls differs in powdered spinach by ingredient form as measured with an *in vitro* gastro-intestinal digestion, anaerobic fecal fermentation model

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Abstract

Insights into food matrix factors impacting bioavailability of bioactive carotenoids and chlorophylls from fruits and vegetable ingredients are essential to understanding their ability to promote health. Stability and bioaccessibility of carotenoids and chlorophylls were assessed from dehydrated, spray-dried, freeze-dried and fresh spinach ingredient forms using *in vitro* models simulating upper gastrointestinal (GI) digestion, lower GI anaerobic fecal fermentation. Intestinal transport of bioaccessible bioactives from both upper and lower GI compartments was assessed using the Caco-2 human intestinal cell model. Differences in carotenoid and chlorophyll content were observed between ingredient forms and these influenced bioaccessibility. Lower carotenoid and chlorophyll content in spray dried spinach resulted in the lowest total bioaccessible content among all spinach treatments (5.8 ± 0.2 μmoles/g DW carotenoid and chlorophyll). Total bioaccessible content was statistically similar between freeze-dried (12.5 ± 0.6 μmoles/g DW), dehydrated (12.5 ± 3.2 μmoles/g DW), and fresh spinach (14.2 ± 1.2 μmoles/g DW). Post anaerobic fermentation, cellular accumulation of carotenoids was higher (17.57-19.52 vs 5.11-8.56%), while chlorophylls were lower (3.05-5.27 vs 5.25-6.44%), compared to those observed following upper GI digestion. Collectively, these data suggest that spinach forms created by various drying technologies deliver similar levels of bioaccessible spinach bioactives and that the lower GI tract may serve as a site for significant absorption fostered by interactions with gut microbial communities that liberate addition bioactives from the spinach matrix.

Keywords: Processing; Vegetable; Bioactives; Bioavailability; Gut Microbial Fermentation

Introduction

The association between dietary patterns rich in fruit and vegetables and risk of chronic and degenerative diseases has been well established.[1] In alignment with these associations, establishment of dietary guidance [2] that include the promotion of fruit and vegetable consumption continues to be a nutritional policy in the United States.[3,4] However, despite efforts to raise awareness and increase fruit and vegetable consumption, dietary patterns have remained rather unchanged with the average American consuming ~1 cup of fruit and 1.7 cups of vegetables of the recommended 1.5-2 cups of fruit and 2-3 cups of vegetables per day.[5] To overcome this gap, it has been proposed that coordinated efforts between food science and nutrition disciplines and industries could be leveraged to foster development of food processing and ingredient technologies that deliver a diverse array of fruit and vegetable products to help consumers achieve the recommendations included in the 2020 Dietary Guidelines for Americans.[6]

Beyond delivering key nutrients/micronutrients, fruits and vegetables remain a critical source of bioactive phytochemicals including, phenolics, carotenoids and chlorophylls. Of these, carotenoids have been heavily investigated for their role in prevention of age-related macular degeneration,[7] cancer,[8] and cardiovascular disease[9] in addition to their pro-vitamin A activity.[10] Chlorophylls, well known for their role in photosynthesis, have been reported to have potential health benefits including cancer preventative activities as well as anti-inflammatory properties driving more recent interest in this class of bioactives.[11,12]

Of the many fruit and vegetable products available to Americans, spinach is one of the richest sources of both carotenoids and chlorophylls. This includes a diverse array of traditional fresh and processed (juiced, blanched/frozen, and canned) products.[13,14] With recent interest in development of new product platforms formulated with vegetable-based ingredients, availability of powdered spinach ingredients has increased.[15,16] These ingredients often composed of whole spinach or fractions are suitable for use in dietary supplements as well as in functional or nutritional food products.[17,18] Interest in powdered ingredients is often driven by their long shelf life, low cost of transportation, sensory properties and diverse product applications. However, unlike traditional fruit and vegetable products, phytochemical stability, density, and nutrient/phytochemical bioavailability are often not characterized across ingredient platforms. Insights into the bioavailability of micronutrients and phytochemicals from spinach ingredients are needed in order

to align ingredient technologies with product formulations that maximize the delivery of spinach micronutrients and phytochemicals.

Differences in spinach processing and the resulting food matrix structures are known to impact the bioavailability of many lipophilic bioactives including carotenoids and chlorophylls.[19–21] Factors that influence the digestive release and micellarization and bioaccessibility of carotenoids and chlorophylls have been reported from a number of *in vitro* models.[22] These include food/meal mineral content,[23] food matrix effects including cell wall integrity and subcellular structures,[24] mastication,[25,26] thermal processing,[27] and genotype.[28] These findings tend to be well aligned and correlated with in vivo studies that report that carotenoid absorption may vary across genotypes, different food processing methods and food matrices with processing having a generally positive effect on overall bioavailability.[20,29]

While promising, the focus of these and many investigations has been on the factors impacting upper GI digestion and absorption. The fate of vegetable derived carotenoids and chlorophylls in the lower GI is poorly understood. Specifically little is known regarding potential fermentation/metabolism of these bioactives by intestinal microbiota and or the extent to which the lower GI may be a site for significant absorption. Sen et al. (2013) reported that colon concentrations of carotenoids determined from biopsies closely resembled serum concentrations but not dietary patterns and suggested the lower gut as a site for potential absorption of carotenoids.[30] Evidence from animal studies also support the notion that lower intestinal absorption may contribute significantly to circulating carotenoids.[31,32] Furthermore, chlorophylls may play a role in altering gut dysbiosis associated with obesity.[33] While limited *in vitro* work suggests that carotenoids may not be completely metabolized by gut microbial communities[34–36], there are reports of chlorophyll metabolism by gut bacterial communities including generation of water soluble pheophorbides which have recently been reported as bioavailable chlorophyll forms in systemic circulation.[37,38] With growing evidence for lower intestinal absorption of carotenoids and chlorophylls, it is important to consider how ingredient technology may impact the release and stability of these bioactives in both upper and lower GI compartments.

To understand these influences, fresh, dehydrated, freeze-dried, and spray dried spinach ingredient forms were generated and used as a model for green vegetables. These ingredient forms were subjected to *in vitro* digestion models simulating both upper (oral, gastric and small intestinal) and lower GI (anaerobic fecal fermentation) with both regions independently coupled

to a Caco-2 human intestinal cell model (intestinal absorption). Through simulated digestion/fermentation of spinach forms, exploration of differences in their bioaccessible fractions and subsequent cellular accumulation were assessed in parallel to provide insight as to how carotenoid and chlorophyll delivery may be impacted by ingredient form.

Methods and Materials

Chemicals and Reagents

Chlorophyll a and b, β-carotene, lutein, and *trans*-β-apo-8'-carotenal authentic standards were obtained from Sigma Aldrich (St. Louis, MO, USA) for calibration, identification, quantification, and determination of extraction efficiency. Chlorophyll a and b were quantitively converted to pheophytin a and b, respectively, for calibration purposes as previously described.[39] ACS and LC/MS grade water, methanol, ethyl acetate, petroleum ether, acetone, methyl-tert-butyl ether (MTBE) were sourced from ThermoFisher (ThermoFisher Scientific, Waltham, MA, USA). α-amylase (A3176, 10 units/mg solid), mucin (M2378), pepsin (P7125), pancreatin (P7545), lipase (L3126), bile (B8631), butylated hydroxy toluene (BHT), and ammonium acetate were sourced from Sigma. Cell culture reagents included fetal bovine serum (Atlanta Biologicals, Flowery Branch, Georgia, USA), HEPES and nonessential amino acids (ThermoFisher Scienific), fatty acid free albumin (Sigma), Dulbecco's Phosphate Buffered Saline (DPBS), gentamicin (Lonza, Basal, Switzerland), penicillin-streptomycin, Dulbecco Modified Eagle Media (DMEM), flasks, and 6-well plates (Corning, Corning, NY, USA). A bicinchoninic acid assay (BCA) kit was sourced from ThermoFisher Scientific. The fermentation media was comprised of resazurin, peptone water, yeast extract, dipotassium phosphate, monopotassium phosphate, magnesium sulfate heptahydrate, calcium chloride hexahydrate, sodium bicarbonate, Tween 80, Hemin, vitamin K1, and L-cysteine all sourced from Sigma Aldrich and bile salts and sodium chloride sourced from ThermoFisher.

Experimental Design

Figure 1 displays an overview of the digestion and fermentation experimental design. Four spinach treatments were processed by *in vitro* digestion simulating oral, gastric, and small intestinal phases of digestion. A portion of the small intestinal digesta was centrifuged and filtered

resulting in an aqueous fraction (upper GI bioaccessible fraction) and undigested residue. A portion of digesta and residue for each were then combined (20:80) for anaerobic fermentation to simulate the proportion of the digested spinach treatment that would reach the colon in vivo. Fermentations were carried out for 48 hours and resulting fermenta was collected at 0, 6, 12, 24, and 48 hours. Both aqueous fractions from upper GI digestion and fermenta fractions collected after 6 hours of fermentation for each treatment were applied to Caco-2 monolayer to determine cellular accumulation of carotenoids and chlorophylls from each phase of digestion.

Spinach Material Preparation

All spinach was sourced from a local supermarket (Green Gate, Costco, Charlotte, NC) on the same day and all leaves used for the experiment were combined and four composite samples were generated, one for each of the four processing treatments: fresh, dehydrated powder, freeze-dried powder, and spray-dried powder. Fresh spinach was stored in the refrigerator for three days prior to being homogenized (8 g of spinach with 8 ml of water) for 30 seconds using a VWR homogenizer, aliquoted, and extracted. Fresh spinach was dehydrated by hot-air drying in a Cosori (Anaheim, CA, USA) dehydrator at 57 °C for 13 hours. Dehydrated spinach was ground using a Cuisinart SG-10 electric grinder (Stamford, Connecticut, US) for 20 seconds and four sets of dehydrated spinach were combined and stored in a vacuum sealed bag at -80 °C until further analysis. Freeze-dried spinach was produced by initially blanching fresh spinach leaves for two minutes at 100 °C and followed by rapid cooling in an ice bath. Blanched spinach was patted dry and stored at -80 °C overnight prior to lyophilization using a SP VirTis freeze dryer (Warminster, PA, USA). Blanched-freeze dried spinach was then ground using a Cuisinart SG-10 electric grinder (Stamford, Connecticut, US) for 20 seconds and stored in a vacuum sealed bag at -80 °C until further analysis. Spinacgh for spray drying was first juiced using a hydraulic cold press juicer (GSE 5300, Tribest, Anaheim, CA). The obtained spinach juice was centrifuged at 4 °C for 20 min then filtered through 6 layers of cheesecloth using a Buchner funnel and filter flask. The resulting extract was then concentrated in a Rotavapor (Buchi Labortechnik AG, Switzerland) until total soluble solids reached 8.0 ± 0.2 ^{0}Brix. The spinach juice concentrate was atomized using a laboratory scale spray dryer (B-290, Buchi Labortechnik AG, Switzerland) operating at inlet temperature 140 °C and outlet temperature ranging from 65-70 °C following preliminary experiments (data not shown). The spray drying system used air in co-current flow with a 1.5 mm

diameter nozzle, and air flow 600 L/h. The feed solution was kept under constant magnetic stirring during drying and the feed flow (controlled by peristaltic pump) was kept at 10 mL/min. The resulting spray dried particles were collected from the collection chamber and weighed. Two independent spray drying batches were combined and stored in a vacuum sealed bag at -80 °C until further analysis.

All powdered spinach forms were sieved and only particles below 250 μm were collected for use. Sieved particles were used to create a slurry by thoroughly mixing 2.5 g of particles with 50 ml of water to be used for *in vitro* analyses. Slurries were then aliquoted and stored at -80°C until used for extractions and digestion. Homogenized fresh spinach along with both powdered and slurry forms of spinach were analyzed in triplicate for moisture content using a CEM Corporation Smart 6 moisture/solids analyzer (Matthews, NC, USA). Water activity of powdered spinach forms was measured in triplicate using an AquaLab Pre water activity meter (Four Marks, Alton, Hants, United Kingdom) at room temperature. The particle size of each powder was determined in quadruplicate via laser diffraction using a Mastersizer 3000 (Malvern Instruments Ltd, Worcestershire, UK) equipped with an Aero S dry powder dispersion unit. The refractive index was set at 1.45 and size measurements were expressed as mean particle diameter. Particle L* a* b* values were determined using a CR-400 Chroma Meter (Konica Minolta, Tokoyo, Japan). Further, bulk density of powdered spinach forms was assessed in triplicate by measuring the mass of powdered spinach that could pack into a 10 ml graduated cylinder.[40] **Table 1** displays all dried spinach particle characteristics.

In vitro Oral, Gastric and Small Intestinal Digestion

All spinach forms were digested using an adapted three-stage *in vitro* digestion method previously described.[41] Briefly, α-amylase (10 units/mg) was added to 2.5 g of each spinach slurry (slurries were created by adding 2.5g of spinach particles to 50 ml of water) with 5% canola oil and incubated for 10 minutes at 37 °C, 120 rpm in a shaking incubator. The volume was adjusted to 30 ml with a 0.9% saline (NaCl) solution, pepsin was added (final concentration of 0.5 g/L), the pH was adjusted to 2.5± 0.1 and the volume was adjusted to 40 ml with 0.9% saline. After 1 hour of incubation at 37 °C, 120 rpm, pH was adjusted to 5± 0.1 prior to small intestinal enzyme addition including pancreatin and lipase (both with a final concentration of 0.8 g/L) and bile (final concentration of 1.8 g/L). The pH was adjusted to 7.0 ± 0.1 after enzyme addition and volume

adjusted to 50 ml with 0.9% saline. Samples were then incubated for 2 hours at 37 °C, 120 rpm. Aliquots of the resulting digesta were saved and additional aliquots were centrifuged at 4 °C and 3,428 × g for 75 minutes in an Eppendorf 5920 R (Hamburg, Germany) centrifuge. After centrifugation, micellarized carotenoids and chlorophylls were isolated by filtration of the supernatant with 0.2 μm cellulose acetate filters. The digesta, filtered supernatant (aqueous phase), and the pellet containing insoluble residue, were all stored at -80 °C prior to additional analysis or further use.

In vitro anaerobic fecal fermentation

The residual pellet resulting from centrifugation of 40 ml of small intestinal digesta was resolubilized in 10 ml of PBS and 8 ml of this mixture was combined with 8 ml of crude digesta (creating a 20:80 ratio, based on the solids content in initial digestion volume) for fermentation. Treatments included the digesta-pellet mix resulting from fresh spinach, dehydrated spinach, freeze-dried spinach, spray-dried spinach, and a spinach extract control (spinach extract was used as a vegetable matrix control and was not digested but prepared as described in supplemental materials). Controls included a fecal-free fresh spinach control (media and fresh spinach), a fecal-free spinach extract control (media and spinach extract), a spinach-free treatment control (fecal slurry and media), and an inulin positive fermentability control (media, fecal slurry and inulin). All treatments were analyzed over the course of two fermentations and the appropriate controls were run in parallel to each spinach fermentation.

Fermentation was carried out as previously reported.[42] Fecal material was sourced from the FMP-R pack from OpenBiome (Cambridge, Massachusetts, USA). Fecal donors were recruited and selected based on a screening excluding participants that did not comply with the 200-point clinical assessment, including but not limited to those with chronic disease, with viral, bacterial and/or parasitic agents, or with abnormal behavioral traits, BMI, or vital signs. Fecal collections were processed in a solution of sterile 12.5% glycerol and 0.9% saline buffer prior to filtration through a 330 μm filter. A fecal slurry, comprising 2% of the fermentation volume, was created by diluting one mL of filtered feces from two different donors by 10-fold with sterile sparged PBS.

Fermentation media was comprised of peptone water (2g), yeast extract (2g), sodium chloride (0.1g), dipotassium phosphate (40mg), disodium phosphate (40mg), magnesium sulfate heptahydrate (10mg), calcium chloride hexahydrate (10mg), sodium bicarbonate (2g), Tween 80

(2mL), Haemin (50mg), vitamin K1 (10µL), and bile salts (0.5g) in 500 mL of DI water. L-cysteine (0.5g) and resazurin (1mg) in 500 mL of DI water were boiled separately until colorless. Both portions of the media were sterile-filtered and flushed with nitrogen overnight. On the day of the fermentation both portions of media were combined 1:1 inside the anaerobic chamber prior to use. In total, fermentation samples contained 16 ml of digesta-pellet mix that was added to 0.84 mL of fecal slurry and 25.16 mL of media resulting in a total fermentation volume of 42 mL.

Fermentation was carried out in a 4-glove anaerobic chamber (855-ACB, Plas-Labs) flushed with a mixed gas comprised of 5% hydrogen, 5% carbon dioxide, and 90% nitrogen. Chamber conditions were monitored during fermentations to maintain a hydrogen gas level between 3.0 and 3.9%, a temperature between 37 and 40 °C, a humidity between 50-70%, and an oxygen level at <15 ppm. The fermentations were initiated by addition of fecal slurry to the media and treatment in each respective tube and the fermentation was carried out for 48 hours. The pH was measured every 4.5 hours using a Metrohm 855 titrator (Riverview, FL, USA) (**Supplemental Table 1**), and aliquots were collected from each fermentation tube at 0, 6, 12, 24, and 48 hours for analysis. A portion of each aliquot was centrifuged at 4 °C and 3,428 × g for 75 minutes in an Eppendorf 5920 R (Hamburg, Germany) centrifuge and both the uncentrifuged and centrifuged fractions were extracted for quantification of carotenoids and chlorophylls. Mean levels of carotenoid and chlorophyll detected in the fecal controls (background) were subtracted from all treatment samples to more accurately reflect carotenoid and chlorophyll content resulting from spinach treatments alone.

Caco-2 Cellular Accumulation

Caco-2 cells, HTB-37 (American Type culture Collection, Rockville, MD, USA) were cultured as previously described.[43] Cells between passages 29-32 were seeded at a density of 1.28 × 10^5 per cm^2, and cultured on 6 well plates using DMEM with 10% (v/v) heat inactivated fetal bovine serum (excepting 20% on days 13-19), 1% penicillin-streptomycin, 1% non-essential amino acids, 1% 1M HEPES, and 0.1% gentamycin in a 37 °C incubator at 5% CO_2, and grown on 6 well plates. 10-11 days post-confluency, experiments were carried out by washing cells with 1 ml 0.1% fatty acid free albumin in DPBS at 37 °C and again with 1 mL of DPBS at 37 °C. Two separate sets of cellular accumulation experiments were conducted, one using the aqueous material (passages 31-32) resulting from the upper GI *in vitro* digestion and another using the fermenta

(passages 29-30) resulting from 6 hours of anaerobic fecal fermentation. Aqueous fraction and bioaccessible fermenta treatments were combined with DMEM at a 1:4 ratio for treatment to the cells. Treatments were applied to the apical surface of the cells for 0, 2, 4, and 6 hours after which cells were rinsed with 1 mL 0.1% fatty acid free albumin in DPBS at 37 °C, rinsed again with 1 mL of DPBS at 37 °C and then followed with ice cold DPBS to help scrape and remove cells from all wells for further analysis. All cells were blanketed with nitrogen and stored in 2 ml vials at -80 °C. To minimize the potential for photooxidation, preparation and treatment was conducted under reduced light to help prevent carotenoid and chlorophyll degradation. Cell-free media control treatments were analyzed to capture degradation of carotenoids and chlorophylls during incubation and used to adjust accumulation results at the respective 0, 2, 4, and 6-hour treatment times. Protein analysis was conducted using a Pierce BCA protein assay kit (ThermoFisher Scientific).

Carotenoid and Chlorophyll Extraction and LC-PDA Analysis

100mg aliquots of freshly homogenized spinach and slurries of all powdered spinach forms were extracted twice with acetone and once with MTBE. All aqueous, digesta, residual pellet mixtures, and fermenta were extracted three times with a 1:3 ratio of acetone: petroleum ether (0.1% BHT). Extraction recoveries from all liquid-liquid extractions as determined by spiking with trans-β-apo-8'-carotenal ranged from 92-100%. Caco-2 cells were thawed in cold water and lysed by sonication (Branson Ultrasonifer 450) for 10 seconds prior to extraction with a 1:3 ratio of acetone: petroleum ether (0.1% BHT) three times. For each procedure, extractions were combined and dried under a stream of N_2, resolubilized in a 1:1 ratio of ethyl acetate: methanol prior to filtration through 0.45 μm PTFE filters and subsequent injection on the LC. Four replicates of each extract were assessed.

The extraction recovery of carotenoid and chlorophylls between raw material extraction methods and liquid-liquid extraction methods was discovered to be different in the case of the pellet matrix. This difference was quantified in the pellet matrix by comparing an extraction of the pellet using the raw material extraction method to an extraction of the pellet resolubolized in DPBS using the liquid-liquid extraction method and the respective correction factor (**Table 2)** applied to the fermenta at time point zero. This correction allowed for the appropriate consideration of matrix effects and determination of changes in carotenoid and chlorophyll concentration throughout fermentation.

All extracts of carotenoids and chlorophylls were quantified using Waters Alliance 2695 LC system (Waters, Milford, MA, USA) equipped with a 2998 Photo Diode Array (PDA) Detector (Milford, MA, USA). A gradient elution method was used to separate carotenoids and chlorophylls on a C18 XSelect HSS T3 3.5 µm, 3 × 150 mm column with a mobile phase A consisting of a ratio of 1:39:60 1M ammonium acetate adjusted to pH 4.6: water: methanol and mobile phase B consisting of ethyl acetate with 0.5% BHT. The gradient started at 70% A and 30% B and transitioned to 50% A and 50% B over 4 minutes, to 25% A and 75% B over the next 8 minutes, to 0% A and 100% B over the following 2 minutes and back to 70% A and 30% B over 5 minutes where it was held for an additional 2 minutes. Quantification of compounds was carried out at 450 nm, excepting pheophorbide a at 650 nm, based on multilevel calibration curves constructed with authentic standards for each respective compound.

Data Analyses

All data are represented as the mean and standard deviation of four replicates for each spinach treatment. The following measures were determined by the respective calculations detailed below. For simplified presentation, individual carotenoid and chlorophyll derivatives are summed and results were presented as totals of carotenoid and chlorophyll classes.

$$\textbf{Small Intestinal Bioaccessibility (\%)} = \frac{aqueous\ bioactive\ content\ (picomoles\ per\ digestion\ volume)}{digesta\ bioactive\ content\ (picomoles\ per\ digestion\ volume)} \times 100$$

Small Intestinal Bioaccessible Content (*µ*moles/g DW) = *Bioaccessibility* × *raw material bioactive content*

$$\textbf{Fermenta Bioaccessibility (\%)} = \frac{supernatant\ of\ fermenta\ (picomoles\ per\ fermentation\ volume)}{fermenta\ (picomoles\ per\ fermentation\ volume)} \times 100$$

Fermenta Bioaccessible Content (*µ*moles/g DW) = *Bioaccessibility at 48 hours* × *bioactive content of digesta-pellet mix measured at 0 hours of fermentation*

$$\textbf{Cellular Uptake Efficiency (\%)} = \frac{cellular\ bioactive\ content\ (adjusted\ for\ stability)}{aqueous\ bioactive\ content} \times 100$$

Cellular Accumulation (pmoles/mg protein) = Cellular bioactive content (adjusted for stability)

All data are presented in moles to be inclusive of all forms and account for conversion of chlorophyll derivatives through digestion. Cellular uptake efficiency and cellular accumulation calculations apply to cell treatments with aqueous material and fermenta material. For determination of significance among treatments within each analysis conducted, SAS JMP (Cary, NC, USA) was used to conduct ANOVA, T-test, and Tukey's *post hoc* analysis ($\alpha = 0.05$).

Results and Discussion

Raw Material Content

Qualitatively, carotenoid and chlorophyll profiles in spinach varied across form (Supplemental Table 2). Quantitatively, expressed on a dry weight basis (DW), freeze-dried spinach retained the highest total carotenoid (3.71 $\pm$ 0.12 μmoles/g) and chlorophyll (24.46 $\pm$ 0.75 μmoles/g) content after processing. Consistent with other reports,[44,45] fresh spinach was lower in carotenoid (2.99 $\pm$ 0.02 μmoles/g) and chlorophyll (19.69 $\pm$ 0.69 μmoles/g) content compared to freeze-dried spinach. This may be due, in part, to differences in extraction efficiency as a result of thermal treatment and matrix disruption. Dehydrated spinach powder had similar chlorophyll content (19.26 $\pm$ 1.09 μmoles/g) to fresh, and a modestly lower carotenoid content (2.62 $\pm$ 0.13 μmoles/g). Spray dried spinach powder had a significantly ($p < 0.05$) lower carotenoid (1.18 $\pm$ 0.04 μmoles/g) and chlorophyll (7.56 $\pm$ 0.15 μmoles/g) content compared to all other treatments (**Table 3)**.

The current data highlight the influence of drying processes such as spray-drying, which have previously been reported to significantly influence carotenoid and chlorophyll content.[46] Also consistent with previous studies, freeze drying had no apparent influence on carotenoid and chlorophyll content as compared to materials that are blanched (spray dryed product).[47,48] Interestingly, dehydration of fresh spinach also provided high recovery for carotenoids (88%) and chlorophyll (98%) supporting the notion that approproiate drying processes can also be used to retain phytochemical.

Carotenoid and Chlorophyll Small Intestinal Bioaccessibility

Bioaccessibility, is a predictor of bioavailability in humans as it refers to the portion of carotenoids and chlorophylls released by digestion and transferred from the food matrix to mixed bile salt lipid micelles for absorption in the small intestine.[49] While, differences exist between spinach treatments in carotenoid and chlorophyll content at the start of the digestion that may impact bioaccessibility, bioaccessibility expressed as a percentage is the key relative measure to compare treatments as it impacts the total overall bioaccessible content directly. (**Table 3**). The relative bioaccessibility total bioactive forms was 43.6±2.5% for spray-dried spinach, 36.3±4.3% for fresh, 34.4±10.6% for dehydrated spinach, and 15.9±4.0% for freeze-dried spinach (**Table 3**). Though higher in relative bioaccessibility, spray-dried spinach had a lower total bioactive bioaccessible content of 3.8±0.2 μmoles/g being similar to freeze-dried spinach, 4.5±1.2 μmoles/g, primarily driven by its lower starting content. Fresh and dehydrated spinach were distinct from the other two treatments having a higher bioaccessible contents of 8.2±1.0 μmoles/g and 7.5±2.3 μmoles/g, respectively (**Table 3**).

Trends in small intestinal bioaccessible content did not align completely with starting material content trends. Rather, fresh and dehydrated spinach deliver significantly more chlorophyll than spray dried and freeze-dried spinach while freeze-dried spinach delivered the most carotenoid followed by fresh, dehydrated, and spray dried spinach (**Table 3**). These findings are consistent with our previous report that thermally treated spinach may result in a lower bioaccessibility due, in part, to the release of divalent minerals through processing and subsequent interference with lipid micellarization [reported as bioaccessibility (%) in Table 3].[26] However, it must also be considered that the physical properties of particles including structure and size could also play a role in modifying susceptibility to digestion and interactions that ultimately impact micellarization.[27,40,50,51] It is plausible then that the improvement in bioaccessibility from spray-dried spinach may be driven by factors such as its reduced smaller particle size or increased porosity and wettability compared to the decreased density and moisture of freeze-dried spinach (**Table 1**).

Fermentation Stability and Bioaccessibility of Carotenoids and Chlorophylls

Carotenoid and chlorophyll contents were also monitored throughout the course of the 48-hour anaerobic fecal fermentation to understand the possible direct and indirect influence of the microbiota and any changes in carotenoid and chlorophyll profiles were driven by microbial

metabolism or chemical degradation under lower GI conditions. **Figures 2a-i** display changes in bioactive content expressed as a percent of starting content to provide relative comparison by treatment. Total carotenoid content as measured from fresh, dehydrated, spray-dried, and freeze-dried spinach was reduced to $74.6 \pm 1.7\%$, $63.3 \pm 3.6\%$, $78.2 \pm 0.9\%$, and $83.8 \pm 3.6\%$, of starting content respectively after 6 hours of anaerobic fecal fermentation. After 6 hours, carotenoid content proceeded to increase such that by the end of the 48-hour fermentation all treatments remained between 80-103% of their original content. Total chlorophyll content for fresh, dehydrated, spray-dried, and freeze-dried spinach, similarly, dropped to $82.0 \pm 1.6\%$, $72.5 \pm 6.7\%$, $85.4 \pm 3.0\%$, and $87.2 \pm 1.0\%$, respectively, of starting content within the first six hours and then increased in content throughout the remainder of the fermentation to result in 79-103% of the original chlorophyll content. The observed decrease followed by an increase in carotenoid and chlorophyll content of digested spinach treatments may be indicative of changes in extractability and/or stability through lower GI digestion/anaerobic fecal fermentation.

A decrease in carotenoid and chlorophyll content was also observed in fecal free spinach under anaerobic conditions, however, this process was slower, over 12 hours rather than 6 hours, suggesting that the presence of fecal microbiota do affect carotenoid and chlorophyll degradation processes and observed rate of loss. Additional control fermentation of spinach extracts (vegetable matrix-free control prepared as described in supplemental data) both with and without fecal slurry remained relatively close to 100% of their original content throughout the entire fermentation, with only lutein decreasing by an appreciable amount (**Figure 2a-i**). These results suggest that differences in observed recovery of carotenoids and chlorophylls are driven in large part by differences in spinach matrix and/or more complex reactions/interactions with microbiota that lead to deterioration of key bioactives coupled with enhanced extractability from the digestible residue. It is plausible to consider that presence of a fermentable polysaccharides, such as pectin in the case of spinach, impacts microbial metabolism and may result in a direct effect on bioactive content. Initial decreases in carotenoids and chlorophylls may in fact be driven by chemical mechanisms with the potential of metabolic products of microbial metabolism compounding these reactions. Subsequent increases in content may be derived from increases in bioaccessibility of carotenoids and chlorophylls due to the breakdown of the matrix in a manner similar to those previously reported.[52–54]

In general, these findings suggest that gut microbial metabolism of carotenoids or chlorophylls may be more limited than previously reported using similar models. Specifically, Serrano et al. reported that after *in vitro* digestion and fermentation of leafy greens, only 2-11% of carotenoids remained available in the large intestine and Kaulmann et al. that fermentation of digested cabbage varieties finding that only 4-25% of carotenoids remained after digestion and fermentation of cabbage varieties, suggesting that a large portion of them were metabolized.[34,36] Among these studies, carotenoids may have been metabolized or degredated by other means. The current fermentation was performed under yellow lights to mitigate possible degredation. More similar to results presented here, Goni et al. reported that after digestion and fermentation of a composite of vegetables, 91% of carotenoids remained in-tact after digestion/fermentation and were available for absorption in the lower gut.[35] Among these reports, no microbial metabolites of carotenoids were identified and thus, any losses may be chemical in nature.

Fermenta Bioaccessibility

Tables 4a-b displays the bioaccessibility of each spinach fermenta treatment over the course of the 48-hour fermentation. It was unclear if absorption of lipophilic compounds in the lower gut necessitates true micelle formation or proceeds through other, similar structures. Consequently, the fermenta supernatant was not filtered through 0.2 μm cellulose acetate filters as is done for determination of the small intestinal bioaccessible fraction (i.e. the aqueous fraction). Rather, solubility, or the content in the supernatant, was simply used as a predictor of fermenta bioaccessibility. Bioaccessibility of carotenoids and chlorophylls increased over the course of anaerobic fecal fermentation. This supports the notion that the increase in bioactive release and solubilization may be due to the breakdown of the spinach matrix through lower intestinal digestion and fermentation. This argument is strengthened by the fact that the matrix-free spinach extract fermenta bioaccessibility remained higher than all other treatments throughout the fermentation and reached at least 100% by 48 hours. However, upon assessment of both the fecal-free fresh and fecal-free extract controls, similar trends are observed (**Table 4a-b**). Therefore, there may also be an increase in the solubility of these bioactives that is derived from the *in vitro* fermentation environment (i.e. the presense of tween, bile salts or residuals enzymes from digesta) rather than the metabolic activity of the microbiota. In spite of these findings, an environmental effect of solubility and a microbial effect on matrix breakdown may both be contributing factors

to the observed bioaccessibility increase over the course of fermentation. Future efforts are needed to delineate the relative contribution of these effects and determine the nature of the structure solubilizing lipophilic compounds in the lower GI.

Caco-2 Cellular Accumulation

In an effort to understand potential differences in intestinal uptake between spinach treatments and between small intestinal and fermenta bioaccessible fractions, the aqueous portion of each digested and fermented spinach treatment was diluted with DMEM at a 1:4 ratio and applied to the apical surface of highly differentiated Caco-2 cell monolayers. Accumulations of carotenoid and chlorophyll derivatives were quantified at 0, 2, 4, and 6 hours and values were adjusted to equate for the degradation of each bioactive determined during the cell free treatment experiment. The cellular uptake efficiency of chlorophylls after 6 hours of treatment ranged from 5.3 $\pm$ 1.4 to 6.4 $\pm$ 1.2 % but did not differ significantly across treatments (**Figure 3a-b**). Lutein uptake efficiency after 6 hours of treatment, however, was slightly higher from freeze dried spinach (8.4 $\pm$ 1.8 %,) compared to fresh (6.2 $\pm$ 1.3 %) which was slightly higher than dehydrated and spray-dried spinach (4.8 $\pm$ 1.6 % and 4.7 $\pm$ 1.0 %, respectively). β-carotene uptake efficiency after 6 hours of treatment was significantly higher from fresh spinach (15.0 $\pm$ 1.4 %) compared to dehydrated, spray-dried, and freeze-dried spinach (7.8 $\pm$ 2.1 %, 8.0 $\pm$ 0.8 % and 9.7 $\pm$ 1.3 %, respectively). Ranges of both carotenoid and chlorophyll uptake efficiency are within ranges reported previously for aqueous fractions of carotenoids and chlorophylls following similar *in vitro* digestion conditions.[36,55,56]

Though years of research have established the absorption pathways of carotenoids and chlorophylls in the small intestine, little is known about carotenoid or chlorophyll absorption from the colon. One study suggested that, carotenoids may be absorbed in the colon after a lycopene corn oil emulsion was directly absorbed from the colon of colostomized rats.[32] Further, Gireesh et al. reported β-carotene to be absorbed proportionally to its treatment concentration by colonic epithelial cells that were cultured from fecal material.[57] In the present study, Caco-2 cells were treated with fermenta from 6-hour collections based on previous reports of carotenoid absorption kinetics in vivo that have found serum carotenoid levels to peak between 4 and 6 hours post consumption.[58] This approach provides a direct comparison of the cellular accumulation of fermenta material to the cellular accumulation of aqueous material resulting from the same

digested spinach treatments. The resulting chlorophyll uptake efficiency after 6 hours differed significantly between spinach fermenta treatments ranging between 3.1 ± 0.5 to 5.3 ± 0.4% and was significantly less ($p<0.05$) than aqueous uptake efficiency for all treatments excepting dehydrated spinach ($p=0.3$) (**Figure 3a-b**). Yet, as compared to the aqueous fraction carotenoid uptake efficiency in the fermenta fraction increased significantly for lutein ($p<0.001$) for all treatments and for β-carotene ($p<0.002$) excepting in freeze dried ($p=0.120$) and fresh spinach ($p=0.158$). Lutein uptake efficiency was greatest for spray-dried spinach (20.9±2.1%) followed by fresh and dehydrated spinach (20.2±1.0 and 18.5±2.0%, respectively) and then freeze-dried spinach (17.3±0.56%). On average, β-carotene accumulation was slightly lower than lutein with fresh and spray-dried spinach having uptake efficiencies of 18.0±3.5% and 17.7±3.5%, respectively, followed by dehydrated, 14.2±0.6%, and then freeze-dried, 10.9±0.6%.

Comparatively, total carotenoid uptake efficiency from fermenta was significantly higher than carotenoid uptake efficiency from small intestinal aqueous fraction, particularly attributed to increased lutein uptake efficiency, across all spinach treatments (**Figure 3a-b**). Presumably, this difference in accumulation may be correlated to a difference in the solubility of carotenoids within the aqueous fraction and fermenta environments as previously discussed and presented in **Tables 3a-b**. Goni et al., hypothesized that modulation of the food matrix by intestinal microbiota may result in increases in the accessibility and/or solubility of these compounds consistent with the increased bioaccessibility of carotenoids through fermentation.[34] However, it cannot go unrecognized that additives including bile salts[57] and surfactants including tweens,[59] both of which were present in the fermentation media, have been used previously to aid carotenoid solubility within an aqueous environment at similar levels.[59,60]

Inconsistent with the solubility hypothesis are the trends evidenced for chlorophyll accumulation. Chlorophyll solubility appeared to increase similarly to carotenoid solubility over the course of the fermentation (**Table 4a-b**), yet the cellular accumulation of these compounds was slightly, though significantly, lower than comparative uptake from aqueous fractions for all treatments excepting dehydrated spinach (**Figure 3a-b**). Therefore, solubility in the lower GI may not necessarily predict chlorophyll intestinal uptake, despite the fact that lipophilic chlorophylls are thought to be absorbed similarly to carotenoids. Thus, the mechanism driving these differences remains to be explored. Yet, these findings do provide additional evidence that carotenoids and

chlorophylls liberated and made bioaccessible through anaerobic fecal fermentation may in fact be absorbable consistent with previous reports.[34,36]

Comparative Assessment of Spinach Treatments

Figures 4a-b illustrate the relative bioaccessibility of each spinach treatment throughout the entire digestive process. It becomes apparent that content, digestion, and fecal fermentation differentially impact total bioaccessibility of each spinach treatment. The relative contribution of small intestinal bioaccessible content to total bioaccessible content was 36% for freeze-dried spinach, while it was 58%, 60% and 66% for fresh, dehydrated, and spray-dried spinach, respectively. Further, as with small intestinal bioaccessibility, it should be considered that the physical characteristics of the particles may differentially influence the fermented bioaccessibility of these treatments. Specifically, when comparing fresh spinach to dehydrated spinach, fermented chlorophyll bioaccessible content contributes to 44% and 40% of total bioaccessible content, respectively, while fermented carotenoid bioaccessible content contributes 26% and 44% to total fermented bioaccessibility, respectively. Thus, the particle formed through dehydration could be responsible for observed difference in carotenoid bioaccessibility of dehydrated spinach relative to fresh spinach through fermentation. It is important to acknowledge that while results presented here may be reflective of the bioaccessible fraction of carotenoids and chlorophylls when particles are consumed in supplement form they may not be indicative of bioaccessibility in the case when particles are included in a formulated food product which could present other matrix effects.[61,62] Additional factors including food matrix effects,[24] the presence of other compounds interfering with or stimulating micellarization,[23,58] and any effects of additional thermal treatment may alter bioaccessibility.[63]

Conclusion

Taken together, these data highlight a broader view of bioaccessibility using *in vitro* models. These data suggest that spray dried spinach may not be as effective in delivery of carotenoids and chlorophylls as compared with other treatments. Blanching, as reported previously, appeared to impede small intestinal bioaccessibility of freeze-dried spinach compared to other treatments but the relative decrease was offset by a relative increase in the fermented bioaccessible content such that the resulting total carotenoid and chlorophyll bioaccessible content

were more reflective of raw material content. The consideration of bioaccessible fraction available after lower GI fermentation, may negate observed differences assessed in the small intestinal bioaccessible compartment. Further, cellular accumulation of carotenoids increased, and chlorophylls decreased, post fermentation as compared to the cellular accumulation of aqueous material. These findings point to the possible role of gut microbial fermentation in the continued breakdown of the food matrix but also highlight the need for a more thorough understanding of the mechanism of absorption in the lower gut and how interactions with microbiota and broader metabolites may influence these processes.

Funding
Funding for this research was provided by a grant from the Foundation for Food and Agriculture Research (FFAR - award #534667). The content of this publication is solely the responsibility of the authors and does not necessarily represent the official views of FFAR. MGF was supported by the United States Department of Agriculture National Institute of Food and Agriculture, Hatch Project NC 02618.

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Figure Captions

Figure 1. Overview of experimental design and methodologies applied for upper GI digestion and lower GI anerobic fecal fermentation. Bioaccessibility was assessed seperatly for both upper and lower GI compartments. Intestinal uptake was assessed from supernatant fractions of digesta and fermenta from upper and lower compartments independently using the Caco-2 human intestinal cell model. Controls for vegetable matrix (extract) and fermentation (inulin) are identified. Four replicates were assessed reach each spinach sample at each stage of the experimental design.

Figure 2. 48h recovery of carotenoids and chlorophylls through anerobic fecal fermentation of lutein (panel a), all chlorophyll b derivatives including: chlorophyll b, chlorophyll b', and others (panel b), total chlorophyll a including: chlorophyll a and all pheophytin a derivatives (panel c), β-carotene (panel d), all pheophytin b derivatives including: pheophytin b and others (panel e), total chlorophyll b including: all chlorophyll b derivatives and all pheophytin b derivatives (panel f), total carotenoid including: lutein and β-carotene (panel g), all pheophytin a derivatives including: pheophytin a and pheophytin a' (panel h), total chlorophyll including: total chlorophyll b and total chlorophyll a (panel i). Data represent a mean ± SD of 4 independent measurements.

Figure 3. 6h cellular uptake of total carotenoid (panel A) and total chlorophyll (panel B) derivatives from media prepared from diluted aqueous bioaccessible fractions from upper GI digesta (white bars) and lower GI fecal fermentation (black bars). Data represent a mean ± SD of 4 independent measurements. Statistical lettering describes the aqueous as compared to the fermenta cellular uptake within each spinach treatment for carotenoids (panel A) and chlorophylls (panel B). Data represent a mean ± SD of 4 independent measurements.

Figure 4. Total bioaccessible content (Small intestinal bioaccessible content + fermented bioaccessible content) derived from each spinach form for carotenoids (panel A) and chlorophylls (panel B). Data represent a mean ± SD of 4 independent measurements. Statistical lettering details the difference in total bioaccessible content among spinach treatments of total carotenoids (panel A) and total chlorophylls (panel B) independently.

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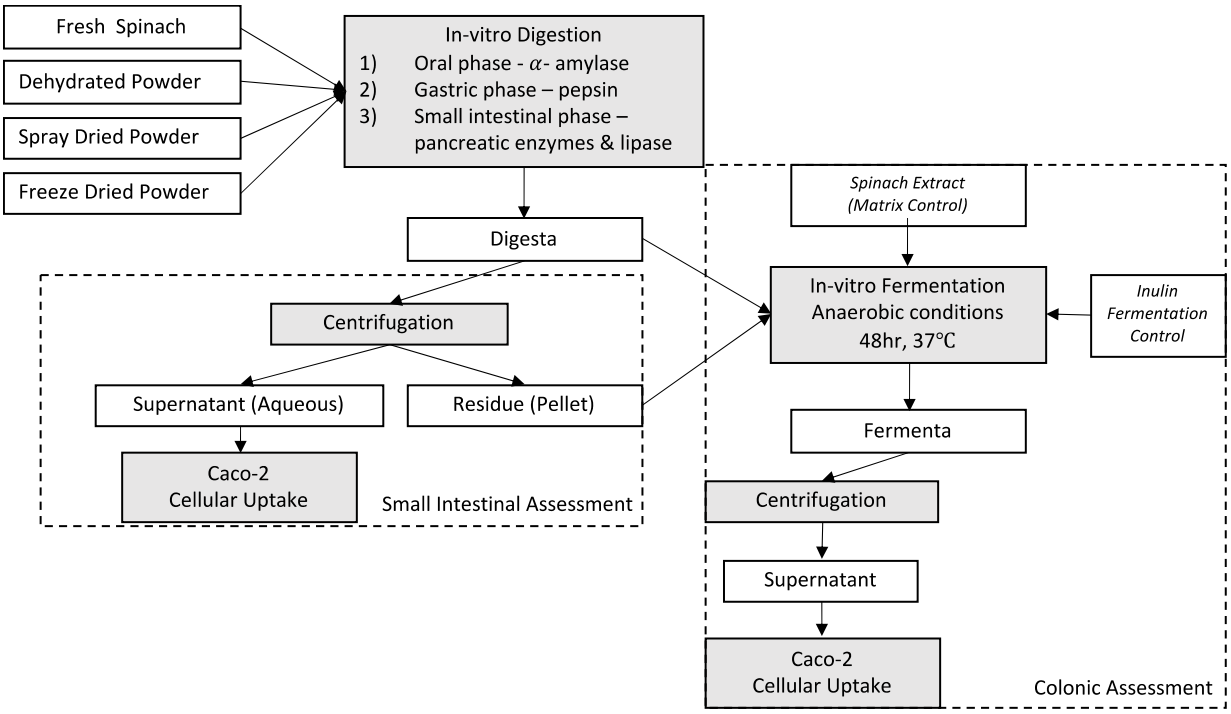
Fresh Spinach
Dehydrated Powder
Spray Dried Powder
Freeze Dried Powder
In-vitro Digestion
1) Oral phase - α- amylase
2) Gastric phase – pepsin
3) Small intestinal phase – pancreatic enzymes & lipase
Digesta
Centrifugation
Supernatant (Aqueous)
Residue (Pellet)
Caco-2 Cellular Uptake
Small Intestinal Assessment
Spinach Extract (Matrix Control)
In-vitro Fermentation Anaerobic conditions 48hr, 37℃
Inulin Fermentation Control
Fermenta
Centrifugation
Supernatant
Caco-2 Cellular Uptake
Colonic Assessment

Figure 1.

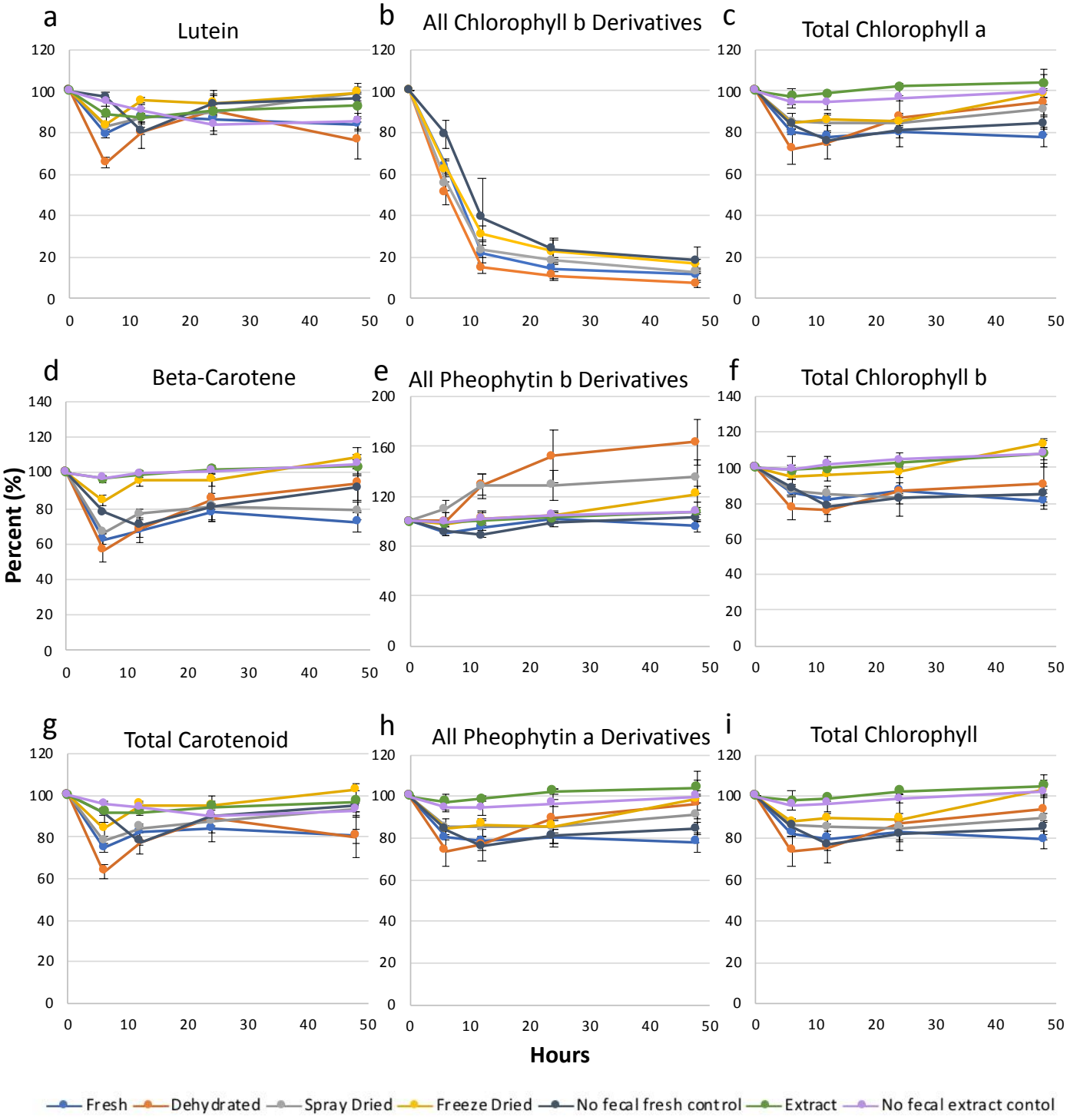
a
Lutein
b
All Chlorophyll b Derivatives
c
Total Chlorophyll a
d
Beta-Carotene
e
All Pheophytin b Derivatives
f
Total Chlorophyll b
g
Total Carotenoid
h
All Pheophytin a Derivatives
i
Total Chlorophyll
Percent (%)
Hours
Fresh
Dehydrated
Spray Dried
Freeze Dried
No fecal fresh control
Extract
No fecal extract contol

Figure 2.

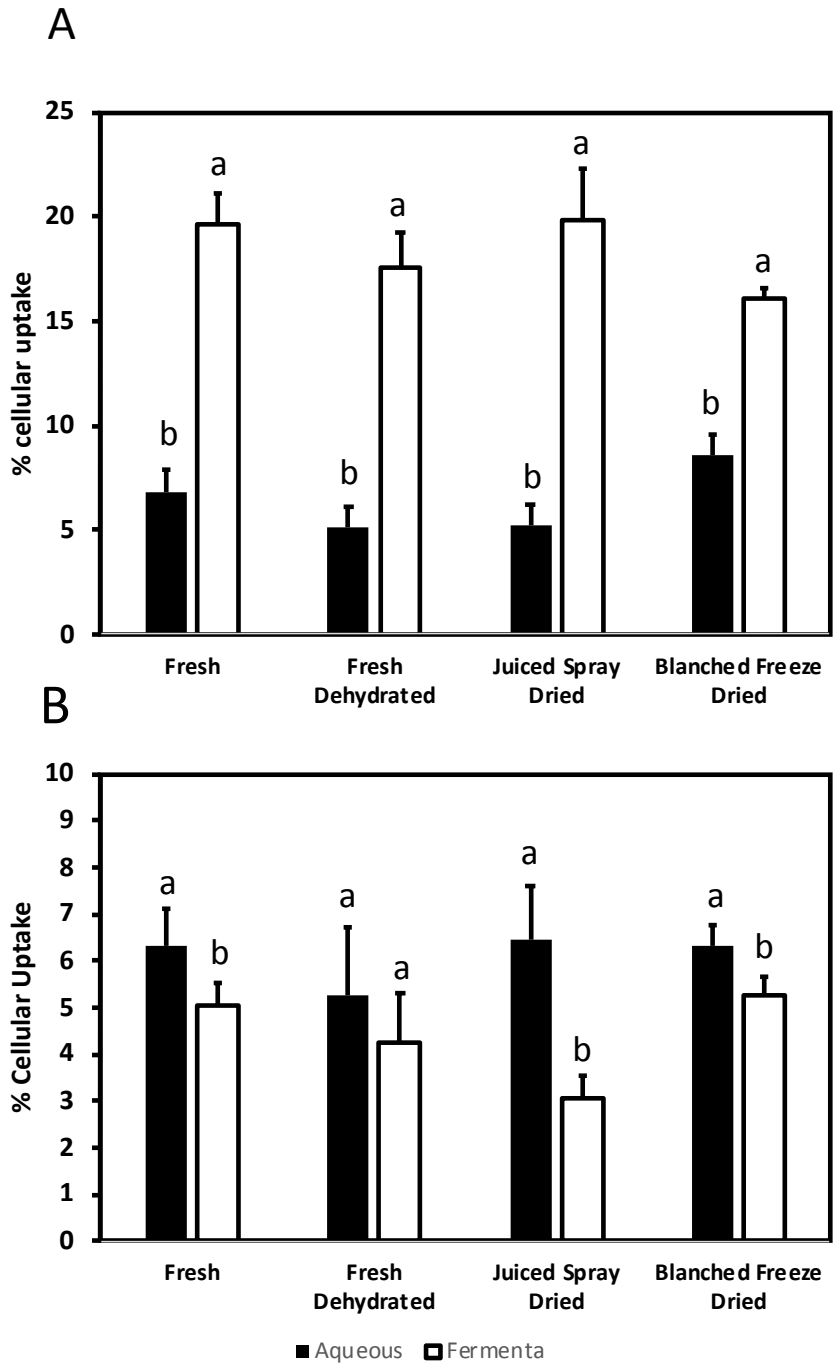
A
% cellular uptake
25
20
15
10
5
0
a
a
a
a
b
b
b
b
Fresh
Fresh Dehydrated
Juiced Spray Dried
Blanched Freeze Dried
B
% Cellular Uptake
10
9
8
7
6
5
4
3
2
1
0
a
b
a
a
a
b
a
b
Fresh
Fresh Dehydrated
Juiced Spray Dried
Blanched Freeze Dried
Aqueous
Fermenta

Figure 3.

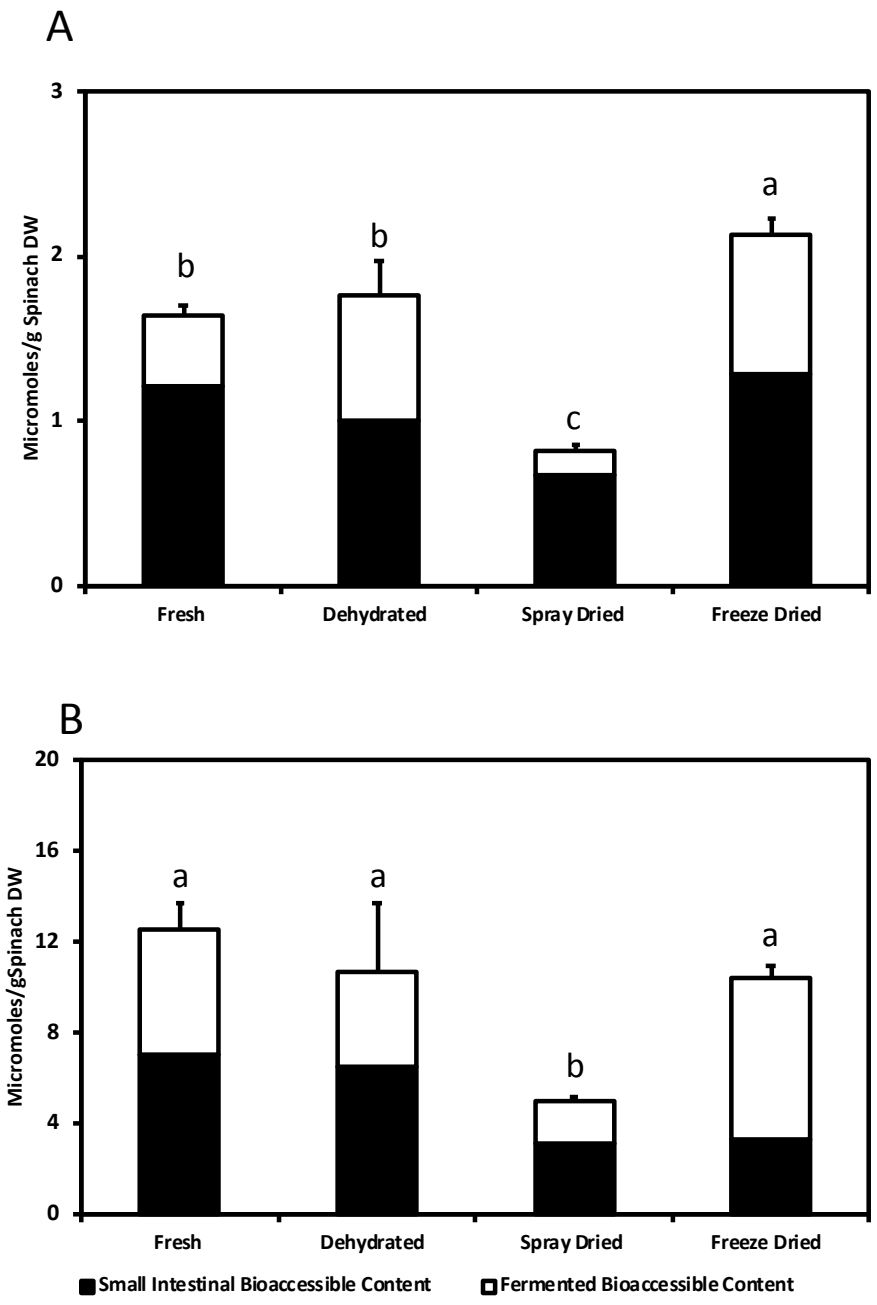
A
Micromoles/g Spinach DW
3
2
1
0
b
b
c
a
Fresh
Dehydrated
Spray Dried
Freeze Dried
B
Micromoles/gSpinach DW
20
16
12
8
4
0
a
a
b
a
Fresh
Dehydrated
Spray Dried
Freeze Dried
Small Intestinal Bioaccessible Content
Fermented Bioaccessible Content

Figure 4.

Table 1: Particle Characterization

Powder	Color			Particle Size Distribution (microns)							Moisture (%)	Water Activity	Bulk Density (g/ml)
	L*	a*	b*	0-10	11-100	101-300	301-500	500-1000	1001-3500	Median			
Fresh-Dehydrated	50.16	-12.34	18.95	11.64	58.54	26.45	3.34	0.02	0	56.23	4.06	0.26	0.46
Blanched-Freeze Dried	48.84	-10.51	14.95	4.64	60.17	29.89	3.39	1.93	0	65.30	2.58	0.12	0.24
Juiced-Spray Dried	61.08	-17.37	33.16	48.83	23.02	6.93	2.97	9.12	9.12	11.60	5.78	0.37	0.48

Table 2: Correction factors used to correct for the difference in the extraction recovery of carotenoids and chlorophylls from liquid-liquid as compared to raw material extraction methods

Sample	Chlorophyll b	Lutein	Pheophytin b	Pheophytin a	β-Carotene
Fresh	1	0.997	0.931	0.868	0.740
Dehydrated	1	1	0.934	0.799	0.685
Spray Dried	1	1	1	0.963	0.840
Freeze Dried	1	1	1	0.917	0.890

Table 3: Comparison of Content, Bioaccessibility, Bioaccessible Content, Aqueous Cellular Uptake Efficiency and Fermenta Cellular Uptake Efficiency across Fresh, Fresh Dehydrated, Juiced Spray-Dried, and Blanched Freeze-Dried Spinach Treatments[1]

	Sample	*Lutein*	*Beta-Carotene*	*Total Chlorophyll a*	*Total Chlorophyll b*	*Total Carotenoid*	*Total Chlorophyll*	*Total Bioactive*
Content (µmoles/g DW)	Fresh	1.78±0.01^{b}	1.21±0.01^{b}	14.85±0.28^{b}	4.84±0.04^{b}	2.99±0.02^{b}	19.69±0.26^{b}	22.69±0.27^{b}
	Dehydrated	1.68±0.09^{b}	0.94±0.04^{c}	14.54±0.84^{b}	4.73±0.24^{b}	2.62±0.13^{c}	19.26±1.09^{b}	21.89±1.21^{b}
	Spray-Dried	0.67±0.02^{c}	0.50±0.02^{d}	5.75±0.10^{c}	1.81±0.06^{c}	1.18±0.04^{d}	7.56±0.15^{c}	8.74±0.18^{c}
	Freeze Dried	2.22±0.07^{a}	1.48±0.04^{a}	18.41±0.58^{a}	6.05±0.17^{a}	3.71±0.12^{a}	24.46±0.75^{a}	28.16±0.87^{a}
Small Intestinal Bioaccessibility (%)	Fresh	49.04±2.72^{b}	19.23±3.19^{bc}	38.54±4.85^{a}	28.87±4.03^{b}	40.42±2.58^{b}	35.8±4.63^{a}	36.28±4.31^{a}
	Dehydrated	42.37±3.13^{b}	27.47±8.91^{ab}	35.75±12.91^{a}	29.23±9.09^{b}	38.35±4.53^{b}	33.76±11.68^{a}	34.38±10.64^{a}
	Spray-Dried	67.41±3.42^{a}	38.20±3.34^{a}	42.17±3.02^{a}	41.35±2.94^{a}	57.12±3.41^{a}	41.94±2.50^{a}	43.62±2.53^{a}
	Freeze Dried	46.09±4.81^{b}	13.66±3.27^{c}	14.37±4.50^{b}	10.52±2.88^{c}	34.52±4.27^{b}	13.38±4.08^{b}	15.86±3.99^{b}
Small Intestinal Bioaccessible Content (µmoles/g DW)	Fresh	0.87±0.05^{ab}	0.23±0.04^{a}	5.73±0.78^{a}	1.40±0.19^{a}	1.21±0.08^{ab}	7.06±0.96^{a}	8.24±1.03^{a}
	Dehydrated	0.71±0.08^{b}	0.26±0.08^{a}	5.19±1.88^{a}	1.38±0.43^{a}	1.01±0.13^{b}	6.49±2.25^{a}	7.52±2.33^{a}
	Spray-Dried	0.45±0.02^{c}	0.19±0.01^{a}	2.42±0.18^{b}	0.75±0.06^{b}	0.67±0.04^{c}	3.17±0.19^{b}	3.81±0.21^{b}
	Freeze Dried	1.02±0.12^{a}	0.20±0.05^{a}	2.66±0.88^{b}	0.64±0.18^{b}	1.28±0.17^{a}	3.28±1.06^{b}	4.48±1.21^{b}
Fermented Bioaccessibility (%)[2]	Fresh	80.97±0.06^{b}	77.35±0.07^{b}	68.55±0.04^{b}	65.61±0.03^{b}	80.06±0.06^{b}	67.67±0.04^{b}	68.44±0.04^{b}
	Dehydrated	74.9±0.03^{b}	69.91±0.01^{b}	54.38±0.03^{c}	52.18±0.01^{c}	73.55±0.02^{bc}	53.73±0.03^{c}	55.74±0.02^{c}
	Spray-Dried	92.06±0.02^{a}	97.08±0.04^{a}	78.53±0.01^{a}	73.14±0.01^{a}	93.28±0.02^{a}	77.03±0.01^{a}	78.13±0.01^{a}
	Freeze Dried	80.99±0.03^{b}	57.69±0.03^{c}	51.51±0.04^{c}	46.28±0.04^{d}	72.10±0.02^{c}	49.92±0.04^{c}	51.60±0.04^{c}
Fermented Bioaccessible Content (µmoles/g DW)	Fresh	0.31±0.02^{b}	0.12±0.01^{b}	3.94±0.26^{b}	1.58±0.15^{ab}	0.43±0.02^{b}	5.52±0.4^{b}	5.94±0.41^{b}
	Dehydrated	0.59±0.09^{a}	0.17±0.03^{b}	2.98±0.58^{b}	1.23±0.23^{b}	0.76±0.12^{a}	4.21±0.81^{b}	4.95±0.93^{b}
	Spray-Dried	0.11±0.00^{c}	0.05±0.00^{c}	1.32±0.03^{c}	0.51±0.01^{c}	0.15±0.01^{c}	1.83±0.03^{c}	1.99±0.03^{c}
	Freeze Dried	0.61±0.06^{a}	0.25±0.04^{a}	5.36±0.91^{a}	1.83±0.28^{a}	0.85±0.11^{a}	7.16±1.19^{a}	8.02±1.29^{a}
Total Bioaccessible Content (µmoles/g DW)[3]	Fresh	1.18±0.05^{b}	0.35±0.04^{ab}	9.67±0.86^{a}	2.97±0.31^{a}	1.64±0.07^{b}	12.58±1.16^{a}	14.18±1.2^{a}
	Dehydrated	1.31±0.11^{b}	0.43±0.11^{a}	8.17±2.44^{a}	2.61±0.63^{a}	1.77±0.2^{b}	10.71±3.02^{a}	12.46±3.2^{a}
	Spray-Dried	0.56±0.02^{c}	0.24±0.01^{b}	3.75±0.18^{b}	1.26±0.06^{b}	0.83±0.03^{c}	5.00±0.17^{b}	5.80±0.19^{b}
	Freeze Dried	1.64±0.07^{a}	0.45±0.03^{a}	8.01±0.38^{a}	2.47±0.15^{a}	2.13±0.10^{a}	10.45±0.51^{a}	12.5±0.57^{a}

[1]Averages and standard deviations are reported from four replicates of each sample (n=4).
[2]Fermented bioaccessible content is calculated based on the bioaccessibility of bioactives after 48 hours of fermentation (bioactives measured in supernatant after 48 hours divided by bioactives measured in fermenta after 48 hours) multiplied by initial bioactive content of the fermentation at 0 hours (comprised of the 20:80 ratio of digesta:pellet used for fermentation).
[3]Total bioaccessible content is the sum of small intestinal and fermented bioaccessible content
Statistical lettering details the difference between each spinach treatment within each bioactive compound for each measue of content and bioaccessitility.

Table 4: Carotenoid and Chlorophyll Fermenta Bioaccessibility (%).

Average Total Carotenoid Bioaccessibility Through Fermentation (%)[1]						
	0hr	**6hr**	**12hr**	**24hr**	**48hr**	**% Increase**
Fresh	54.5±3.7	53.0±2.8	54.5±1.8	64.3±8.1	80.1±6.5	**25.6**
Dehydrated	31.8±2.7	42.0±1.7	40.3±3.6	43.5±2.7	73.6±2.2	**41.8**
Spray Dried	78.7±2.7	77.0±1.6	77.0±1.6	75.9±0.7	93.3±2.1	**14.9**
Freeze Dried	46.5±3.6	52.1±5.0	50.5±3.4	57.0±6.6	72.1±1.6	**25.6**
Extract	83.9±0.8	95.6±1.2	94.3±2.6	95.5±4.3	103.9±4.3	**20.0**
Fecal free fresh control	57.7±2.4	58.3±0.3	54.2±2.0	59.1±1.7	75.0±2.2	**17.3**
Fecal free extract control	86.1±4.1	92.2±1.5	87.1±1.8	92.6±1.1	102.8±1.1	**16.8**

Average Total Chlorophyll Bioaccessibility Through Fermentation (%)[1]						
	0hr	**6hr**	**12hr**	**24hr**	**48hr**	**% Increase**
Fresh	40.8±2.1	46.7±4.3	51.0±1.7	57.7±3.5	67.7±3.8	**26.8**
Dehydrated	29.9±3.6	36.8±1.2	38.2±2.0	40.4±1.3	53.7±2.7	**22.8**
Spray Dried	46.3±0.2	48.5±1.4	50.2±3.6	54.0±0.8	77.0±1.1	**30.7**
Freeze Dried	31.5±6.9	36.8±7.1	40.0±5.3	442±6.7	49.9±3.9	**18.5**
Extract	72.0±3.6	94.9±4.4	97.8±1.7	94.9±4.4	106.4±4.3	**34.3**
Fecal free fresh control	42.8±1.1	48.5±3.6	49.0±5.7	55.2±2.7	65.6±6.2	**22.8**
Fecal free extract control	65.6±10.4	82.7±6.5	80.7±6.1	90.7±5.3	99.9±1.0	**34.3**

[1]Averages and standard deviations are reported from four replicates of each sample (n=4).