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Iron and Zinc Bioavailabilities to Pigs from Red and White Beans (*Phaseolus vulgaris* L.) Are Similar

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Common beans contain relatively high concentrations of iron (Fe) and zinc (Zn) but are also high in polyphenols and phytates, factors that may inhibit Fe and Zn absorption. In vitro (Caco-2 cells) and in vivo (pigs) models were used to compare Fe and Zn bioavailabilities between red and white beans, which differ in polyphenol content. Bean/maize diets containing 37% of either white or red cooked beans were formulated. Fe uptake by Caco-2 cells was 14-fold higher from the white bean diet compared to the red bean diet. The diets were fed to anemic piglets ($n = 10$) for 35 days. On experiment days 7 and 21, pigs were given meals containing beans intrinsically labeled with stable isotopes of Fe and Zn (^{58}Fe, ^{70}Zn), followed by intravenous (iv) injections of ^{54}Fe and ^{67}Zn, to assess Fe and Zn absorption. Isotope ratios determined by inductively coupled plasma mass spectrometry in whole blood and plasma samples were used to calculate iron and zinc absorption, respectively. On day 35, animals were killed and duodenal sections were collected for DMT1 gene expression analysis. Fe absorption was 14 and 16% from the first labeled meal and 9 and 10.5% from the second labeled meal for the white and red beans, respectively ($P > 0.05$). Zn absorption was 28 and 23% from the first meal ($P > 0.05$) and 31 and 29% from the second meal ($P > 0.05$) for the white and red beans, respectively. DMT1 gene expression did not differ between treatments. It was concluded that bean color does not affect Fe or Zn bioavailability in vivo and that beans are a good source of bioavailable Fe and Zn.

KEYWORDS: Beans; iron; zinc; bioavailability; absorption; pigs; stable isotopes; polyphenols

INTRODUCTION

Iron deficiency affects approximately 2 billion people worldwide (*1*), and nearly a fourth of the world's population may have inadequate intakes of bioavailable zinc (*2*). A major cause of Fe and Zn deficiencies is low bioavailability from plant-based diets containing mineral absorption inhibitors such as polyphenols and phytates.

Common beans (*Phaseolus vulgaris* L.) are dietary staples in many regions of the world. They are good sources of protein, energy, minerals, and vitamins (*2*). Beans are especially rich in iron, containing between 50 and 150 mg of Fe/kg (*3*). In addition, bean consumption may reduce the risk for some chronic diseases including coronary heart disease, diabetes, and obesity (*2*).

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Beans are also rich in polyphenols and phytates (*4*), components that may inhibit the bioavailability of minerals such as Fe and Zn (*5*). Polyphenols are concentrated in the seed coats of beans (*6*). In general, colored beans contain higher concentrations of flavanoids than white beans (*4*). Thus, if polyphenols do inhibit iron and zinc absorption, it is reasonable to expect that bioavailability from colored beans would be lower than that from white beans.

In vitro studies using a Caco-2 cell model have repeatedly shown that iron bioavailability from white beans is significantly higher than that from colored beans (*4, 6*). In contrast, iron absorptions by women (*7*) and piglets (*8*) from red and white beans were similar. The effect of polyphenols on Zn bioavailability, if any, is largely unstudied, although Ganji and Kies (*9*) reported that tea, a beverage rich in polyphenols, did not have a significant effect on zinc absorption in healthy human subjects.

The objective of the present study was to compare Fe and Zn bioavailabilities between diets containing either white (Matterhorn) or red (Merlot) beans. Young anemic pigs were fed a meal containing intrinsically labeled (^{58}Fe, ^{70}Zn) beans

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Table 1. Composition of Experimental Diets

ingredient	g/kg diet (by formulation)	
	white bean diet	red bean diet
Matterhorn beans	370	
Merlot beans		370
maize, yellow dent	432	432
whey protein isolate[a]	120	120
maize oil	20	20
vitamin premix2	10	10
mineral premix (no Fe)[b]	45	45
L-lysine	1	1
DL-methionine	1	1
L-threonine	1	1
Tylan[c]	5	5
total	1000	1000
selected components by analysis, means ± SE		
Fe, mg/kg of diet[d]	50.2 ± 1.2	42.4 ± 0.9
Zn, mg/kg of diet[d]	83.8 ± 1.8	85.2 ± 1.6
total phenols, μg/g of diet	76.4 ± 4.1	104.3 ± 4.8
phytate, μmol/g of diet	9.43 ± 0.396	9.93 ± 0.07

[a] Ultra 8000-Grade A (Grande Custom Ingredients Group, Lomira, WI). [b] Vitamin and mineral premixes were purchased from Dyets, Inc., Bethlehem, PA. They were formulated to provide the following concentrations (per kg of diet): thiamin−HCl, 1 mg; riboflavin, 3.75 mg; pyridoxine−HCl, 1 mg; niacin, 10 mg; calcium pantothenate, 12 mg; folic acid, 1.3 mg; biotin 0.2 mg; vitamin B-12, 15 μg; retinol palmitate, 8 mg; vitamin D-3, 0.5 mg; α-tocopheryl acetate, 88 mg; menadione sodium bisulfite, 0.8 mg; Ca, 7.5 g; P (available), 3.5 g; Na, 1.5 g, Cl, 1.5 g; Mg, 0.4 g; K, 2.04 g; Cu, 5 mg; I, 0.13 mg; Fe, 0 mg; Se, 0.14 mg; Zn, 50 mg. [c] Tylan is a macrolide antibiotic used as a growth promoter in commercial swine operations, Eli Lilly Co. [d] Iron and zinc concentrations in the diets were determined by an inductively coupled argon-plasma/atomic emission spectrophotometer (ICAP 61E Thermal Jarrell Ash Trace Analyzer, Jarrell Ash Co., Franklin, MA) following wet ashing.

followed by an intravenous (iv) injection of ^{54}Fe and ^{67}Zn. Absorption of iron and Zn was calculated from isotope ratios in whole blood and plasma, respectively.

MATERIALS AND METHODS

Experimental Animals and Protocols. Weanling crossbred pigs (Yorkshire × Hamshire × Landrace) from the Cornell University Swine Farm were used. All animal protocols were approved by the Cornell University Institutional Animal Care and Use Committee. Piglets were selected from litters that were given an intramuscular injection of 50 mg of Fe as iron-dextran at birth, half of the dose used in commercial swine operations, so that they would be Fe deficient at weaning. After weaning, the piglets were allocated to two treatment groups ($n = 10$) on the basis of body weight (mean of 7.70 ± 0.19 kg), litter, gender, and hemoglobin concentration. Pigs were housed individually in stainless steel cages in a temperature-controlled barn (22−25 °C) with a light/dark cycle of 12/12 h, given free access to feed and water, and checked daily. After 5 weeks, pigs were killed, and sections (5 cm) of the small intestine were quickly removed and frozen in liquid nitrogen.

Diets, Study Design, and Blood Sample Collection. Two bean varieties (*P. vulgaris* L.), Matterhorn Great Northern (white) and Merlot small red (red), were used (**Table 1**). Beans were mixed with water (∼1 kg of beans/1.5 L of water) and cooked in an autoclave at 121 °C for 30 min. Following cooking, they were freeze-dried and ground to a coarse powder for incorporation into diets. Diets contained no supplemental Fe but were otherwise adequate in protein, essential fatty acids, vitamins, and minerals for pigs of this age. On days 7 and 21 of the experimental period, pigs were given a meal containing beans intrinsically labeled with enriched stable isotopes of Fe(III) and Zn(II) (^{58}Fe, ^{70}Zn). These meals were identical to what the pigs were given daily except that labeled beans replaced the unlabeled beans. Approximately 3 h after the meals were offered, an intravenous (iv) infusion of ^{54}Fe and ^{67}Zn was administered. Pigs were fed the experimental diets for 5 weeks. Blood samples were collected weekly from the anterior vena cava, using 6 mL Vacutainer heparin-coated tubes (volume collected was ∼4 mL), in the morning following an 8 h overnight fast. The samples were analyzed for hemoglobin (Hb) concentration and Fe and Zn isotope ratios. Additional blood samples (for plasma extractions) were collected 2 days post each administration for the Zn absorption analysis (volume collected was ∼2 mL).

Intrinsically Labeled Beans. White- and red-colored bean seeds of the Matterhorn and Merlot varieties were obtained from Michigan State University. They were germinated in the dark and then transferred to pots and grown in modified Johnson's nutrient solution with the following composition (in mmol/L): N, 16; K, 6; P, 2; Mg, 1; S, 1; Ca, 4; Cl, 0.05; B, 0.0125; Mn, 0.002; Zn, 0.002; Cu, 0.00005; Mo, 0.00001; Ni, 0.00001; Fe, 0.05 [as the Fe(III)-HBED {*N*,*N*′-bis(2-hydroxybenzyl)ethylenediamine-*N*,*N*′-diacetic acid hydrochloride dihydrate} chelate]. At flowering, Zn and Fe were supplied as $^{70}ZnCl_2$ and ^{58}Fe-HBED (^{58}Fe was enriched to 92.8% and ^{70}Zn to 99.533%) (Isoflex USA, San Francisco,CA) with final concentrations in the nutrition solution of 2 and 10 μmol/L, respectively. These solutions were supplied after flowering to the plants for 5 weeks (nutrient solutions were changed once a week). When the seeds reached maturity, they were harvested, separated from the pods, autoclaved (in deionized water) at 121 °C for 15 min, homogenized, and lyophilized to dryness. Samples of the dried seed homogenates were wet-digested in a two-step process, first with concentrated HNO_3 and second with a 50:50 mixture of HNO_3 + $HClO_4$. The digests were diluted with 2% nitric acid solution and analyzed for mineral elements using inductively coupled plasma atomic emission spectrometry (ICP-MS, Agilent 7500cs, Wilmington, DE) with appropriate standards and reference materials as described previously (*10*). Concentrations of both *myo*-inositolpentaphosphate (IP5) and *myo*-inositolhexaphosphate (i.e., phytate; IP6) in acid extracts of samples of dried beans were determined using a liquid ion chromatography method (*11*). Concentrations of Zn, Fe, and phytate in the bean seeds are shown in **Table 2**.

Preparation of Zn and Fe Isotope Solutions for Intravenous Infusion. Enriched stable isotopes of Zn and Fe (^{67}Zn, 94.6% enriched, and ^{54}Fe 99.84%, enriched) (Isoflex USA) were used for this study. The ^{67}ZnO was dissolved in concentrated HCl (Fisher) (5.5 μL of HCl/mg of ^{67}Zn as oxide). The solution was diluted with sterile isotonic saline to a final concentration of 1 mg of ^{67}Zn/mL. The ^{54}Fe (in the form of elemental iron powder) was dissolved in concentrated HCl (Fisher) (200 μL of HCl/mg of Fe). The solution was diluted with triply deionized water to a final concentration of 5 mg of ^{54}Fe/mL (III). For each animal, a volume of 0.2 mL of the ^{54}Fe solution was transferred into a separate vial with 1 mL of the ^{67}Zn solution. The stable isotope solutions were sterilized by filtration and aliquots were stored in sealed, sterile vials until use.

Measurement of Iron Absorption. Iron absorption was estimated from the incorporation of the stable isotope tracers (^{54}Fe from the iv injection and ^{58}Fe from the intrinsically labeled meal) into red blood cells collected 14 days after each administration of the isotopes. Blood samples (0.2 mL) were placed in 20 mL quartz tubes and wet-digested in 1 mL of concentrated HNO_3 followed by a 50:50 mixture of HNO_3 + $HClO_4$ (0.5 mL) and brought to near dryness in a heating block. The ash was dissolved in 15 mL of 2% (0.3 mol/L) nitric acid, and isotope ratios were determined via ICP-MS (Agilent 7500 CS). The amounts of Fe stable isotopes (^{54}Fe or ^{58}Fe) in excess of the naturally occurring amounts in the total circulating hemoglobin of the animals were calculated from mass spectrometer isotope ratio analyses of whole blood, Hb concentration measurements, and estimates of blood volume (*12*). The amount of stable isotope tracer incorporated into hemoglobin was calculated as follows (*13*):

$$^{58}\mathrm{Fe}_{\mathrm{inc}}\,(\mathrm{mg}) = \frac{[^{58}\mathrm{Fe}/^{56}\mathrm{Fe}_{\mathrm{post}} - {}^{58}\mathrm{Fe}/^{56}\mathrm{Fe}_{\mathrm{pre}}] \times \mathrm{Fe}_{\mathrm{circ}}\,(\mathrm{mg}) \times \mathrm{NA}_{58}}{^{58}\mathrm{Fe}/^{56}\mathrm{Fe}_{\mathrm{pre}}}$$

where $^{58}Fe_{inc}$ (mg) is the mass in mg of the administered tracer present in the circulating hemoglobin; $^{58}Fe/^{56}Fe_{post}$ is the isotope ratio in

Table 2. Concentrations of Zn, Fe, and Phytate in Bean Seeds[a]

	Zn, μg/g	Fe, μg/g	IP5, μmol/g	IP6 phytate, μmol/g	IP5 + IP6[b], μmol/g
white bean, soil grown (nonlabeled)	25.00 ± 0.176	86.00 ± 1.151	0.46 ± 0.015	8.86 ± 0.087	9.33 ± 0.152
red bean, soil grown (nonlabeled)	30.00 ± 0.145	78.80 ± 0.185	0.43 ± 0.134	8.16 ± 0.204	8.59 ± 0.204
white bean, solution grown (labeled)	29.90 ± 0.04	66.20 ± 0.163	0.96 ± 0.027	14.06 ± 0.3	15.02 ± 0.32
red bean, solution grown (labeled)	34.90 ± 0.100	70.00 ± 0.00	0.7 ± 0.2	13.03 ± 0.34	13.73 ± 0.11

[a] Values are means ± SEM (n=3). [b] IP5 + IP6 = concentration of *myo*-inositol pentaphosphoric acid plus phytate.

Table 3. TaqMan Primers and Probes Used in This Study

target	forward primer[b]	TaqMan probe[c]	reverse primer 2
DMT1[a]	GCAGGTCAGAGCTCCACCAT	ACAGGAACCTACTCCGG-MGB	GAATCCCTCCATGACGAACTG
18S	ACCTACGGAAACCTTGTTACGACTT	CCTCTAGATAGTCAAGTTC-MGB	GCGGAGCGCTGAGAAGAC

[a] Sus scrofa partial mRNA for divalent metal transporter 1 (DMT1 gene, gil86197473l) (*37*). [b] All sequences are presented 5′ to 3′. [c] All probes have a 5′ FAM reporter dye.

circulating hemoglobin Fe 14 days following the administration of the tracer; $^{58}Fe/^{56}Fe_{pre}$ is the isotope ratio in circulating hemoglobin Fe just prior to the administration of the tracer; Fe_{circ}(mg) is the mass in mg of total circulating hemoglobin Fe; and NA_{58} is the natural abundance of ^{58}Fe (0.00287, mole fraction). The same formula was used to calculate ^{54}Fe incorporation using $^{54}Fe/^{56}Fe$ ratios and the natural abundance for ^{54}Fe (0.0584, mole fraction). Circulating hemoglobin iron (Fe_{circ}) was calculated as

$$Fe_{circ} = \text{BW (kg)} \times 0.067 \text{ L of blood/kg of BW} \times [\text{Hb}] \text{ (g/L)} \times 3.35 \text{ mg of Fe/g of Hb}$$

where BW = body weight and [Hb] = hemoglobin concentration.

The percentage of orally administered isotope incorporated into hemoglobin was calculated as

$$^{58}Fe_{inc}\,(\%) = \frac{^{58}Fe_{inc}}{^{58}Fe\text{ (oral)}} \times 100$$

A similar calculation was used to determine the percentage of intravenously administered ^{54}Fe incorporated into hemoglobin. The absorption of the oral dose (^{58}Fe) was then calculated as $^{58}Fe_{inc}$ (%) divided by the percentage incorporated $^{54}Fe \times 100$, as shown below:

$$^{58}Fe_{abs}\,(\%) = {^{58}Fe_{inc}}\,(\%) \times \frac{100}{^{54}Fe_{inc}\,(\%)}$$

Measurement of Zinc Absorption. Fractional absorption of Zn was calculated from isotope ratio analyses on plasma samples prepared from blood drawn 2 days after the iv administration of ^{67}Zn. Plasma samples were digested in nitric/perchloric acid and diluted in 0.3 mol/L nitric acid. Isotope ratios were determined by ICP-MS. The percent excess (%XS) over the naturally occurring amount of each tracer isotope in the plasma was calculated from isotope ratio values obtained from ICP-MS as follows (*14*):

$$\%XS\ ^{70}Zn = \frac{^{70}Zn/^{66}Zn\text{ (post)} - {^{70}Zn/^{66}Zn}\text{ (pre)}}{^{70}Zn/^{66}Zn\text{ (pre)}} \times 100$$

where $^{70}Zn/^{66}Zn$ (post) is the isotope ratio of ^{70}Zn to ^{66}Zn in the plasma after feeding the labeled beans and $^{70}Zn/^{66}Zn$ (pre) is the ratio before feeding the labeled beans. The same formula was used to calculate %XS ^{67}Zn. Percent absorption of the ^{70}Zn from the labeled beans was then calculated as follows (*14*):

$$\%Abs\ ^{70}Zn = \frac{\%XS\ ^{70}Zn}{\%XS\ ^{67}Zn} \times \frac{^{67}Zn\text{ dose (mg)}}{^{70}Zn\text{ dose (mg)}} \times \frac{NA\ ^{70}Zn}{NA\ ^{67}Zn} \times 100\%$$

where %Abs ^{70}Zn is the percent of the ingested ^{70}Zn that was absorbed, NA ^{70}Zn is the natural abundance of ^{70}Zn (0.0062, mole fraction), and NA^{67} Zn is the natural abundance of ^{67}Zn (0.041, mole fraction).

Isolation of Total RNA. Total RNA was extracted from 30 mg of the distal duodenal tissue using Qiagen RNeasy Mini Kit (RNeasy Mini Kit, Qiagen Inc., Valencia, CA) according to the manufacturer's protocol. Briefly, tissues were disrupted and homogenized with a rotor-stator homogenizer in buffer RLT (for lysis of cells/tissues before RNA isolation), containing β-mercaptoethanol. The tissue lysate was centrifuged for 3 min at 8000g in a microcentrifuge. An aliquot of the supernatant was transferred to another tube, combined with 1 volume of 70% ethanol, and mixed immediately. Each sample (700 μL) was applied to an RNeasy mini column and centrifuged for 15 s at 8000g, and the flow-through material was discarded. Next, the RNeasy columns were transferred to new 2 mL collection tubes, and 500 μL of buffer RPE was pipetted onto the RNeasy column followed by centrifugation for 15 s at 8000g. An additional 500 μL of buffer RPE was pipetted onto the RNeasy column and centrifuged for 2 min at 8000g. Total RNA was eluted in 50 μL of RNase free water. All steps were carried out under RNase free conditions. RNA was quantified by absorbance at $A_{260/280}$. Integrity of the 28S and 18 S rRNAs was verified by 1.5% agarose gel electrophoresis followed by ethidium bromide staining. DNA contamination was removed using TURBO DNase treatment and removal kit from AMBION (Austin, TX).

DMT1 Gene Expression Analysis. DMT1 mRNA levels in duodenal mucosa were analyzed by quantitative real-time RT-PCR; values were normalized to 18S expression. Briefly, TaqMan primers and probes were designed using Primer Express software (Applied Biosystems) (**Table 3**). Probes with MGB quencher dye were synthesized by Applied Biosystems. The qRT-PCR was performed in 25 μL reactions (total volume) as previously described (*15*), and 25 ng of total RNA was used per reaction. All reactions were performed in duplicate on at least three independent RNA preparations. A genomic DNA curve was generated for each set of TaqMan reactions as previously described to allow for absolute quantification of mRNA levels (*15*). As also previously described (*15*), transcript levels for each gene were determined as the difference between the experimental reactions and the corresponding reverse-transcriptase negative controls. If the difference between the experimental reactions and the corresponding reverse-transcriptase negative controls was less than twice the value of the reverse-transcriptase negative control, the reaction transcript level was considered to be equal to that of the negative control. This "flooring" approach is based on the ability of the ABI PRISM 7000 and the TaqMan system to detect a minimum 2-fold difference in mRNA levels (ABI PRISM 7000 Sequence Detection System Specifications, Publication 117SP03-04, Applied Biosystems).

In Vitro Iron Bioaccessibility Assessment. An in vitro digestion/Caco-2 cell culture model as described by Glahn et al. (*16*) was used to assess iron bioaccessibility. With this method, foods or meals are subjected to simulated gastric and intestinal digestion. The intestinal digestion is carried out in cylindrical inserts closed on the bottom by a semipermeable membrane and placed in wells containing Caco-2 cell

monolayers bathed in culture medium. The upper chamber was formed by fitting the bottom of a Transwell insert ring (Corning) with a 15000 Da molecular weight cutoff (MWCO) membrane (Spectra/Por 2.1, Spectrum Medical, Gardena, CA). The dialysis membrane was held in place using a silicone ring (Web Seal, Rochester, NY). Iron uptake by the cell monolayers is assessed by measuring ferritin concentrations in the cells. Six replicates of each Fe bioaccessibility measurement were performed.

Briefly, Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD) at passage 17 and used in experiments at passage 29. Cells were seeded at densities of 50000 cells/cm^2 in collagen-treated 6-well plates (Costar Corp., Cambridge, MA). The integrity of the monolayer was verified by optical microscopy. The cells were cultured at 37 °C in an incubator with 5% CO_2 and 95% air atmosphere at constant humidity, and the medium was changed every 48 h. The cells were maintained in Dulbecco's modified Eagle medium plus 1% antibiotic/antimycotic solution, 25 mmol/L HEPES, and 10% fetal bovine serum. Forty-eight hours prior to the experiment, the growth medium was removed from culture wells, the cell layer was washed, and the growth medium was replaced with minimum essential media (MEM) at pH 7.0. The MEM was supplemented with 10 mmol/L PIPES, 1% antibiotic/antimycotic solution, 4 mg/L hydrocortisone, 5 mg/L insulin, 5 μg/L selenium, 34 μg/L triiodothyronine, and 20 μg/L epidermal growth factor. This enriched MEM contained <80 μg iron/L. All ingredients and supplements for cell culture media were obtained from Gibco (Rockville, MD). The cells were used in the iron uptake experiment at 13 days postseeding. In these conditions, the amount of cell protein measured in each well was highly consistent from well to well. On experiment day, 1.5 mL of the digested sample was added to the insert's upper chamber and incubated for 2 h. Then, inserts were removed and 1 mL of MEM was added. Cell cultures were incubated for 22 h at 37 °C.

Harvesting of Caco-2 Cells for Ferritin Analysis. The protocols used in the ferritin and total protein contents analyses of Caco-2 cells were similar to those described by Etcheverry et al. (*17*). Briefly, growth medium was first removed from the culture well by aspiration, and the cells were washed twice with a solution containing 140 mmol/L NaCl, 5 mmol/L KCl, and 10 mmol/L PIPES at pH 7.0. The cells were harvested by adding an aliquot of deionized water and placing them in a sonicator (Lab-line Instruments, Merlose Park, IL). The ferritin and total protein concentrations were determined on an aliquot of the harvested cell suspension with a one-stage sandwich immunoradiometric assay (FER-IRON II Ferritin assay, Ramco Laboratories, Houston, TX) and a colorimetric assay (Bio-Rad DC Protein assay, Bio-Rad, Hercules, CA), respectively. Caco-2 cells synthesize ferritin in response to increases in intracellular iron concentration. Therefore, we used the ratio of ferritin/total protein (expressed as ng of ferritin/mg of protein) as an index of the cellular Fe uptake.

Hemoglobin Analysis. Blood hemoglobin (Hb) concentrations were measured spectrophotometrically using the cyanomethemoglobin method (Pointe Scientific Inc., Canton, MI) following the manufacturer's instructions. Hemoglobin repletion efficiency (HRE) values were calculated as described by Yasuda et al. (*18*).

Serum Iron Analysis. Blood samples for serum Fe analyses were collected (first and last days of the study) using 6 mL serum clot activator tubes (Vacuette, Monroe, NC). Serum was separated by centrifugation (2000*g* at 5 °C for 10 min). Serum Fe concentrations were measured spectrophotometrically using a kit and following the manufacturer's instructions (Serum Iron Reagent, Raichem, CA).

Polyphenol Concentrations in the Beans and Diets. Samples (1.5 g) of cooked white and red beans and the diets were extracted with 4 mL of acidified methanol (methanol and 1.0 M HCl, 85:15 v/v). The samples were shaken for 2 h, vortexed to mix, and centrifuged at 12800*g* for 10 min to remove insoluble material.

To evaluate the total phenols content in the extracts, the method described by Dewanto et al. was used (*19*). Briefly, to 0.125 mL of the supernatant were added 0.5 mL of deionized water and 0.125 mL of the Folin−Ciocalteu reagent. The mixture was allowed to stand for 5 min, and then 0.125 mL of aqueous Na_2CO_3 (7 w%/v) was added. The final volume of the mixture was adjusted to 3 mL with deionized water and was allowed to stand for 90 min at room temperature. The absorbance was measured at 760 nm against a reagent blank. The amount of total phenolics was expressed as gallic acid equivalents (μg/g of sample).

Concentrations of selected polyphenols in the diet extracts were analyzed by high-pressure liquid chromatography using the method described by Espinosa-Alonso et al. (*20*) with slight modifications. The analysis was carried out on a HPLC system (Waters, Milford, MA) consisting of a 600E multisolvent pump, a 717 plus autosampler, and a 996 photodiode array detector set at 264 nm, operated using Empower software. The separation was performed on a Vydac 5u 300A C18 column (Phenomenex), 250 × 4.6 mm. The column was equilibrated using deionized water adjusted to pH 2.8 with glacial acetic acid for 10 min. The extracts were filtered through a 0.20 μm filter (MillexGN, Millipore), and 75 μL of the filtrates was injected into the HPLC system programmed to run a gradient. The gradient program started with 100% of solvent A (deionized water adjusted to pH 2.8 with glacial acetic acid). Solvent B (70:30 v/v, water/acetronitrile; HPLC grade) was increased linearly to reach 10% in 2.5 min, 12% over the next 3.5 min, 23% over the next 10 min, 35% over the next 6 min, and 95% over the next 2 min. The mobile phase was maintained at 95% solvent B for the remaining 6 min. UV absorbance at 282 nm was used to detect flavanoids (kaempferol and astragalin). Total running time of each sample was 40 min.

Phytate Content in Beans and Diets. A Dionex liquid chromatograph system (AS50 autosampler), equipped with conductivity detector model ED50, and gradient pump GS50 were used along with an IonPac AG11 guard column and IonPac AS11 column (4 × 250 mm) to quantify phytate. PeakNet 6.40 software was used to process chromatographic data. The mobile phases were (A) 200 mmol/L NaOH (carbonate-free) and (B) deionized water, using a flow rate of 1 mL/min. Phytate was extracted from 250 mg of dry, lyophilized diet sample, in 10 mL of a 1.25% H_2SO_4 solution; the extraction process was 2 h, after which the samples were centrifuged at 3660*g* for 10 min. Subsamples were diluted 1:10 with deionized water, and 10 μL was injected and analyzed.

Statistical Analysis. One-tailed Student's *t* tests were performed to compare differences between means using the JMP software (SAS Institute, Cary, NC). Values were considered to be significantly different at $P < 0.05$.

RESULTS

Caco-2 Cell Ferritin Formation. Ferritin formation was 67.1 ± 7.31 ng/mg of protein in cells exposed to digests of the white bean diet compared to 4.91 ± 0.49 ng of ferritin/mg of protein in the red beans diet ($P < 0.05$).

Growth Performance, Hemoglobin (Hb) Concentrations, and Hb Repletion Efficiency (HRE). Mean values are presented in **Table 4**. There was no difference in overall growth performance between pigs fed red or white bean diets. HRE values across treatments were not significantly different from each other. Pigs fed the red bean diet had 7% higher Hb concentrations at the end of the study than pigs fed the white bean diet (98.8 ± 4.2 versus 92.4 ± 5.5 g/L, respectively), but the difference was not statistically significant. Similarly, pigs fed the red bean diet had higher overall HRE than pigs fed the white bean diet (23.5 ± 1.2 versus 20.8 ± 1.6%, respectively), but this difference was also not significant.

Iron Absorption from Labeled Meals. Iron absorption was 14.4 ± 1.6 and 16 ± 2.3% from the first meal and 9.8 ± 1.8 and 10.9 ± 1.7% from the second meal for the white and red beans, respectively. There were no significant differences in Fe absorption between the white and red bean meals either when the first meal administration was compared with the second meal administration or between the first and second administrations ($P > 0.05$) (**Table 4**).

Zinc Absorption from Labeled Meals. Zn absorption was 28.1 ± 4.5 and 23.5 ± 3.8% from the first meal and 31.8 ± 3.9 and 29.5 ± 4.2% from the second meal for the white and red

Table 4. Body Weights, Fe Status [Hemoglobin Concentrations [Hb], Hemoglobin Repletion Efficiencies (HRE)], Feed Intake and Fe and Zn Absorption in Piglets Fed Red or White Bean Diets[a]

	treatment	day 0	day 7[b]	day 21[b]	day 35
body wt, kg	WB diet	7.70 ± 0.50	9.7 ± 1.7	14.09 ± 1.85	21.5 ± 3.2
	RB diet	7.70 ± 0.50	10.1 ± 1.6	15.05 ± 2.1	22.7 ± 3.5
[Hb], g/L	WB diet	88.1 ± 2.8	89.8 ± 3.4	91.7 ± 4.7	92.4 ± 5.5
	RB diet	89.2 ± 3.6	93 ± 3.1	94.01 ± 5.1	99.8 ± 4.2
HRE,[c] %	WB diet		22.9 ± 3.5	17.2 ± 2.6	20.8 ± 1.6
	RB diet		28.3 ± 4.3	19.8 ± 2.9	23.5 ± 1.2
daily feed intake (weekly mean), g	WB diet		0.65 ± 0.15	0.996 ± 0.25	1.742 ± 0.44
	RB diet		0.69 ± 0.2	1.155 ± 0.32	1.767 ± 0.47
^{54}Fe (iv) recovery, %	WB diet		93.1 ± 12.2	91.2 ± 11.6	
	RB diet		82.1 ± 8.3	86.1 ± 9.5	
Fe absorption,[d] %	WB diet		14.4 ± 1.6	9.8 ± 1.8	
	RB diet		16.2 ± 2.3	10.9 ± 1.7	
Zn absorption,[e] %	WB diet		28.1 ± 4.5	23.5 ± 3.8	
	RB diet		31.8 ± 3.9	29.5 ± 4.2	

[a] Means ± SE, $n = 10$. Within a time period, there were no significant differences between the red and white bean groups for body weight, Hb, or HRE. WB diet, Great Matterhorn (white diet); RB diet, Merlot (red diet). $P > 0.05$. [b] Stable isotope administration (1 mg of each) was conducted on experiment days 7 and 21. [c] HRE values on days 7, 21, and 35 are cumulative from day 0; for example, the value on day 35 represents absorption over the entire 5 week feeding period. [d] For Fe absorption analysis, we used blood samples that were taken 14 days post each administration of the stable isotopes (i.e., experiment days 21 and 35). [e] For Zn absorption analysis, we used plasma samples that were collected 2 days post each administration of the stable isotopes (i.e., experiment days 9 and 23).

beans, respectively. There were no significant differences in Zn absorption between the white and red bean meals either when the first meal administration was compared with the second meal administration or between the first and second administrations ($P > 0.05$) (**Table 4**).

Pig Duodenal DMT1 Gene Expression. Real-time RT-PCR analysis showed no significant differences ($P > 0.05$) in DMT1 gene expression between bean treatments. The expression levels of the target gene (DMT1 relative to 18S) in samples taken from the duodenum were 1.65 ± 0.25 and 1.71 ± 0.42 for white bean and red bean diets, respectively ($P > 0.05$).

Total Phenolic Concentration in the Bean and Diet Samples. The total phenolics in the bean samples are expressed as gallic acid equivalents (μg/g of sample bean, mean ± STD, $n = 3$) and were 292.1 ± 6.6 and 97.2 ± 2.0 μg/g for the red and white bean samples, respectively. Total phenolics concentrations in the diet samples were 104.4 ± 4.8 and 76.4 ± 4.1 μg/g of diet in the red and white bean diets, respectively ($P \leq 0.05$).

In general, concentrations of specific phenolic acids and flavanoids were similar in the two diets. However, kaempferol and astragalin were found in the red bean diet (7.0 ± 0.6 and 55.3 ± 8.9 μg/g of diet, respectively) but not in the white bean diet.

Serum Fe Concentrations. Serum Fe concentrations were measured at the beginning and at the end of the study. There were no significant differences in serum-Fe concentrations in samples taken from the two treatment groups (values at the end of the study were 98.6 ± 10.5 vs 92.9 ± 11.2 μg/dL in pigs fed the red bean and white bean diets, respectively).

Phytate/Fe Molar Ratios. The concentrations of Fe and phytate in the bean-based diets were used to calculate the phytate to Fe molar ratios. The molar ratios of phytate/Fe were 10.41 ± 0.44 and 13.08 ± 0.08 for the white and red bean diets, respectively ($P > 0.05$).

DISCUSSION

This study confirms and extends our previous finding (*8*) that bean color and polyphenol content do not affect Fe bioavailability from beans in a piglet model and that Fe uptake by Caco-2 cells from white beans is greater than that from red beans. In the piglets, Fe bioavailability, whether assessed by a hemoglobin repletion assay or by the use of stable isotope tracers, was virtually identical in the red bean and white bean diets. Furthermore, the present study is the first to show that Zn bioavailabilities from white and red beans are similar.

HRE represents the percentage of ingested Fe that is incorporated into hemoglobin. Over the 35 day feeding period, HRE values were 20.8 ± 1.6 and 23.5 ± 1.2% for the white and red bean diets, respectively ($P > 0.05$). Fe absorption determined by stable isotope incorporation into hemoglobin was only 14.4 ± 1.6 and 16.2 ± 2.3% for the white and red bean diets ($P > 0.05$) for the isotope doses given on day 7 and 9.8 ± 1.8 and 10.9 ± 1.7 ($P > 0.05$) for the doses given on day 21. The lower absorption seen with the isotopes may be due to lower absorption of bean Fe compared to maize Fe (maize contributed approximately 30% of the Fe in the diets). HRE measures absorption of all the Fe in the diet, whereas only the beans were intrinsically labeled with ^{58}Fe. This would suggest that there is not complete isotopic exchange between the bean Fe and the maize Fe. It is notable that the incorporation of the iv administered ^{54}Fe in hemoglobin Fe ranged from 82 to 93%. This is very close to the 86% average incorporation of iv administered ^{58}Fe seen in a recent study of nonanemic women with low iron stores (*21*).

Very few studies on Zn absorption from beans have been published. Donangelo et al. (*22*) reported Zn absorption of 13−16% by young women from two genotypes of common beans. Sandstrom et al. (*23*) compared Zn absorption by human subjects from meals containing white beans with or without various protein sources (fish, chicken, beef) using an extrinsic radio Zn tag and whole body counting. Zn absorption ranged from 19 to 32%, and the protein source had only a small effect on percentage absorption, Our pigs absorbed between 23 and 32% (mean values) of the intrinsic Zn labels, which compares quite favorably with the values reported by Donangelo and Sandstrom. Kannan et al. (*24*) observed Zn absorptions ranging from 57 to 64% by rats fed bean and bean−rice weaning foods, suggesting that rats are more efficient at absorbing Zn than either humans or pigs.

The lack of an apparent inhibitory effect of polyphenols in our piglet model is puzzling in light of our Caco-2 results and the widely held belief that polyphenols are potent inhibitors of iron absorption. Possible explanations for our results include

the nature of the piglet model, the length of the experimental feeding period, adaptation of the animals to chronic exposure to polyphenols, the source and chemical form of the polyphenols (bean polyphenols differ from tea polyphenols, for example), and the design of the experiment (5 week trial as opposed to a single meal isotope tracer study).

Evidence that polyphenols inhibit Fe absorption comes mainly from studies using tea, a beverage that is rich in polyphenols (green tea contains between 37 and 56% polyphenols on a dry weight basis) (*25*). A typical design for these studies is to label a meal with a radioactive or stable Fe tracer, administer the labeled meal with either tea or water to human subjects following an overnight fast, and measure the concentration of the tracer in red blood cells 2 weeks after administration of the meal. Using this approach, Disler et al. (*26*) reported that tea inhibited Fe absorption from a bread meal by 68%. Hurrell et al. (*27*) reported that tea inhibited Fe absorption from a bread meal by 50−90%, depending on the kind of tea and the concentration of polyphenols in the brewed tea. Thankachan et al. (*28*) compared iron absorption by iron deficient and iron adequate women from a rice meal given with or without 1 or 2 cups of black tea. The concentration of polyphenols in the tea was 78 mg of gallic acid equiv per cup. Tea inhibited iron absorption by a similar amount in both groups of women, and 2 cups of tea inhibited more strongly than 1 cup. Thus, studies of this design consistently show that tea is a potent inhibitor of Fe absorption, presumably due to the high polyphenol concentrations in brewed tea. These studies have led many to hypothesize that consuming tea and other foods high in polyphenols will lead to poor iron status in populations for which these dietary practices are common.

A recent study on the effects of tea polyphenols (epigallocatechin-3-galate and grape seed extract) on Fe uptake and transfer by Caco-2 cells yielded some surprising results that may have relevance to the present study (*29*). In this study, polyphenols enhanced uptake of Fe across the apical brush border of the cells and almost completely blocked transfer of Fe across the basolateral membrane. This effect occurred without changes in cell ferritin, ferroportin-1, divalent metal transporter-1, and transferrin receptor-1 protein. This study suggests that it is possible that the seed coat polyphenols of the pinto beans may block ferritin formation in the Caco-2 cells, which was the measure of cell Fe uptake in the present study. Thus, Fe uptakes from the beans and diets may have been the same but did not stimulate ferritin formation. Further research is warranted to determine if polyphenols block ferritin formation from bean Fe uptake.

Evidence from other phenol-containing foods is less consistent. Tuntipopipat et al. (*30*) compared the effects on Fe absorption of chili powder and turmeric added to a rice-based meal fed to young women, using a stable isotope tag method. Chili powder reduced Fe absorption by 38% but turmeric had no effect, even though it contained twice the amount of phenolic compounds as the chili (50 vs 25 mg). Beiseigel et al. (*7*) compared iron absorption from pinto and white beans in women subjects using an extrinsic radio iron tag method. Iron absorption was similar (about 2%) from the pinto and white varieties even though, presumably, the pinto beans were higher in polyphenols. These two studies suggest that the food source of polyphenols may be an important factor in determining whether or not iron absorption is affected.

Epidemiological studies on the effect of tea on iron status in human populations, for the most part, do not support the hypothesis that tea has a negative effect on iron status. In a cross-sectional study of 954 men and 1639 women, Mennen et al. (*31*) found no relationship between serum ferritin and consumption of black, green, and herbal tea in French adults. In a similar study, Hogenkamp et al. (*32*) did not find an association between black tea consumption and hemoglobin and serum ferritin concentrations in adult South Africans. Mehta et al. (*33*) reported that consumption of tea and coffee was negatively associated with risk for anemia in participants in the NHANES II in the United States; that is, people who drank more tea and coffee had a lower risk for anemia. In a cross-sectional study of 904 preschool children, Gibson (*34*) reported a weak but significant inverse association between tea consumption and serum ferritin ($r = -0.09$, $P < 0.007$). However, the authors acknowledge that British children in this age group consume very little tea.

The apparent lack of an association between tea consumption and iron status in several epidemiological studies is surprising given the consistent and strong inhibitory effect of tea on iron absorption seen in single-meal, isotope tracer studies. It may be that people adapt to high intakes of polyphenols when foods containing them are consumed on a regular basis. A possible mechanism for this adaptation is the synthesis and secretion of proline-rich proteins (PRPs) in the saliva. PRPs have a high affinity for tannins. Presumably, these salivary PRPs play a defensive role against the toxic effects of tannins by precipitating them and preventing them from binding to digestive enzymes and other functional proteins further down in the gastrointestinal tract (*35*). Tannin ingestion up-regulates the synthesis and secretion of tannins by parotid and submandibular glands in rats and mice (*25*). We showed that feeding diets containing tea solids or gelatin (a PRP) to rats for 5 days dramatically reduced the inhibitory effect of tea on Fe absorption (*36*). The concentration of PRP-encoding mRNA in the salivary gland of pigs fed a diet containing red beans was double that of pigs fed a white bean diet (*8*). Whether this up-regulation occurs in humans is unknown, but if it does, regular consumption of tea may not inhibit Fe absorption to the same extent seen in single-meal isotope tracer studies. Therefore, because this study raises questions about the single-meal testing approach, which was the basis of previous studies showing tea inhibition affect on Fe absorption, it would be interesting to test the long-term affect of tea consumption on Fe absorption.

In summary, neither bean color nor bean polyphenol content affected Fe and Zn absorption from maize−bean diets. Moreover, the bioavailability of bean Fe and Zn to pigs is quite good despite high phytate concentrations in the diets. We conclude that common beans are good sources of bioavailable Fe and Zn. An efficacy trial comparing white and red beans in a human population is needed to confirm the findings reported here.

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