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ARTICLE

Dietary Inulin Supplementation Does Not Promote Colonic Iron Absorption in a Porcine Model

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Prebiotics may enhance iron bioavailability by increasing iron absorption in the colon. Anemic pigs fitted with cecal cannulas were fed a low-iron diet with or without 4% inulin. Over 7 days, pigs were administered 1 mg of ^{54}Fe in the morning feed followed by cannula infusion of 0.5 mg of ^{58}Fe to measure total and colonic iron absorption, respectively. Whole blood was drawn prior to the initial dosing and 14 days thereafter for hemoglobin concentration and stable isotope ratio analyses. The prebiotic role of inulin was confirmed by increases in lactobacilli and bifidobacteria with reductions in clostridia using terminal restriction fragment length polymorphism (TRFLP). Total iron absorption was 23.2 ± 2.7 and 20.7 ± 3.5% (mean ± SEM; $p > 0.05$), while colonic iron absorption was 0.4 ± 0.1 and 1.0 ± 0.2% (mean ± SEM; $p > 0.05$) in inulin-fed and control pigs, respectively. These results show that the colon does not make a significant contribution to total iron absorption in iron-deficient pigs and that inulin does not affect iron absorption in the colon.

KEYWORDS: Inulin; prebiotics; iron absorption; pig; stable isotopes

INTRODUCTION

Iron deficiency is currently the most prevalent nutritional deficiency worldwide (*1*, *2*) and is associated with impaired physical work performance, poor immune function, impaired cognitive development, poor pregnancy outcomes, and possibly irreversible developmental delays in infants and toddlers (*2*−*6*). While many factors can contribute to iron deficiency, low bioavailability of dietary iron is widely considered to be a major cause (*3*, *7*, *8*). Currently, food fortification and distribution of iron supplements are the most effective strategies for combating iron deficiency (*9*, *10*). However, compliance in taking iron supplements is often poor because of gastrointestinal upset. Also, low bioavailability from diets high in phytates and polyphenols may limit the effectiveness of fortification. Therefore, increasing the bioavailability of intrinsic dietary iron rather than fortifying food or giving iron supplements may represent a more viable alternative. Recent reports have suggested that prebiotics may enhance iron absorption (*3*, *11*).

Prebiotics, such as inulin, are nondigestible carbohydrates that, in humans, pass through the stomach and small intestine largely undigested and accumulate in the large intestine, where they promote a favorable enteric microbiota through the selective enhancement of beneficial bacterial populations, such as bifidobacteria and lactobacilli, at the expense of pathogenic or opportunistic populations, such as clostridia, enterobacteria, and proteolytic bacteroides species (*12*, *13*). Prebiotics are currently being advocated as a therapeutic/preventative measure for many intestinal and extra intestinal diseases and disorders, including inflammatory bowel disease, diarrhea, and metabolic syndrome, and may also have applications in mineral nutrition (*14*−*17*).

While several studies have demonstrated an enhancing effect of inulin and fructo-oligosaccharide prebiotics on calcium absorption, their impact on iron absorption remains to be fully elucidated (*18*−*21*). Because the prebiotic effect is mediated through microbial fermentation in the large intestine, we hypothesized that inulin enhances iron bioavailability by increasing iron absorption in the large intestine. We therefore used a porcine model to determine the contribution of the small and large intestine to iron absorption from diets with or without supplemental inulin.

MATERIALS AND METHODS

All experiments were performed according to National Research Council (NRC) guidelines and with prior approval of the Cornell University Institutional Animal Care and Use Committee (Protocol 2005-0089).

Experimental Diets. **Table 1** details the composition of the experimental diets. The basal diet was a corn−soy ration replete in all nutrients according to NRC recommendations for pigs (*22*), with the exception of iron, because no inorganic iron was added to the diet. The composition of the inulin diet was identical to that of the basal diet, with the following exception: inulin (Raftilose Synergy I, Orafti) was added at a dose of 40 g/kg diet at the expense of corn starch, which was present at this level in the basal diet.

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Table 1. Composition of the Experimental Diets

ingredient	basal (control) diet	inulin diet
	(g/kg diet)	
corn	620.5	620.5
soybean meal	260	260
corn oil	10	10
corn starch	40	0
inulin	0	40
mineral premix[a]	45	45
vitamin premix[b]	10	10
plasma, spray-dried	10	10
L-lysine	2.5	2.5
D,L-methionine	1.0	1.0
L-threonine	1.0	1.0
total	1000	1000
	(mg/kg diet)	
Fe calculated from formulation	63.0	63.0
Fe by analysis[c]		
experiment 1	84.5 ± 2.2	85.3 ± 1.5
experiment 2		67.4 ± 1.7

[a] Mineral premix (Dyets 295002) provided/kg diet: 12.54 g of $CaCO_3$, 8.42 g of $CaPO_4 \cdot 2H_2O$, 3.81 g of NaCl, 6.96 g of KH_2PO_4, 1.98 g of $MgSO_4$, 40 mg of $MnCO_3$, 80 mg of $ZnCO_3$, 10 mg of cupric carbonate, 200 μg of KIO_3, 300 μg of sodium selenite, and 11.16 g of sucrose. [b] Vitamin premix (Dyets 390020) provided/kg diet: 1 mg of thiamine HCl, 3.8 mg of riboflavin, 1 mg of pyridoxine HCl (vitamin B6), 10 mg of niacin, 12 mg of calcium pantothenate, 1.3 mg of folic acid, 200 μg of biotin, 15 mg of vitamin B12 (0.1%), 8 mg of vitamin A palmitate (500 000 IU/g), 500 μg of vitamin D3 (400 000 IU/g), 88 mg of vitamin E acetate (500 IU/g), 800 μg of menadione sodium bisulfite, and 9.8585 g of sucrose. [c] Analyzed using an ICAP 61E trace analyzer (Thermo Corporation, Waltham, MA).

Raftilose Synergy 1 (Orafti, Belgium) is an inulin product composed of a mixture of α-D-glucopyranosyl-(β-D-fructofuranosyl)$_{n-1}$-β-D-fructofuranoside (n = 10−60, mean of 25) and α-D-fructopyranosyl-(β-D-fructofuranosyl)$_{n-1}$-β-D-fructofuranoside (n = 2−7, mean of 4) (*11*).

Experiment 1: Cannulation Study. A total of 10 castrated, 4-week-old male weaner piglets (Yorkshire × Hampshire × Duroc) were randomly selected from litters at the Cornell University Swine Farm. All piglets had been administered a reduced intramuscular iron dextran injection at birth (50 mg of iron instead of 100 mg) to induce iron deficiency. Pigs were then randomly assigned to one of two treatment groups (control or inulin) according to body weight, such that the between treatment group weight variation was less than 1% of the entire group mean. Pigs were housed individually in stainless-steel metabolism cages and maintained at 22−25 °C with a 12 h light-dark cycle. Water was provided *ad libitum*, while diets were administered twice daily close to the *ad libitum* feed intake, around 5.5% of the body weight at each feed (*23*). Pigs were maintained on their respective diets for a total of 5 weeks, with control pigs receiving the basal diet and inulin pigs receiving the inulin diet. Feed and water intake was monitored on a daily basis, while animal weights were determined weekly. Feces were also scored on a daily basis according to the criteria established by Shu and colleagues (*24*): 1, hard and formed pellets; 2, nonformed pellets; 3, soft feces; 4, very soft containing a small amount of waterlike feces; 5, semisolid containing more than half waterlike feces; 6, waterlike feces.

During the first 3 weeks of feeding, pigs were surgically fitted with cecal cannulas [Percutaneous Endoscopic Gastrostomy (PEG) kit, 20 French, Mila International, Erlanger, KY]. Because of time limitations in the surgery room, all pigs could not be fitted with cannulas on the same day. To eliminate any effect of surgery date, one inulin pig and one control pig were processed on each occasion. Anesthesia was induced with intramuscular administration of Midazolam (0.2 mg/kg) and Ketamine (5 mg/kg) and maintained with sevoflurane. The apex of the cecum was externalized through a 10 cm abdominal incision for cannula placement. The cannula was exteriorized through a second 3 cm incision in the abdomen and anchored to the skin with a plastic fixation device (component of the PEG kit). Pigs were fitted with stockinette vests to protect the suture sites and to prevent cannula displacement. All pigs received a pre- and post-operative dose of analgesic (Flunixin meglumine, 1.1 mg/kg) intravenously (IV) and antibiotic (Ceftiofur Sodium, 5 mg/kg) IV. No further treatments were required because all surgeries proceeded without complication and all piglets recovered well and exhibited normal activity and feed/water intake within 24 h post-operatively.

On 7 consecutive days in week 4, pigs were administered an oral dose of 1 mg of ^{54}Fe (99.84% enrichment; Isoflex, CA) solubilized in 0.01 mol/L HCl mixed in the morning feed and followed 6 h later by 0.5 mg of ^{58}Fe (92.8% enrichment; Isoflex, CA) solubilized in 0.01 mol/L HCl infused into the cecal cannula. Complete consumption of the oral dose was ensured by administering the dose in a small portion of the morning feed. After consumption of this small amount, pigs were then given the remainder of their morning feed. The oral and cecal doses were designed to measure total and colonic iron absorption, respectively. Small intestinal iron absorption was calculated by subtracting the large intestinal absorption from that of the whole intestine.

Whole blood was drawn from the anterior vena cava prior to the initial stable isotope dosing and 14 days thereafter for stable isotope detection and hemoglobin measurement to calculate hemoglobin repletion efficiency (HRE). Serum was also collected at the final blood draw for measurement of serum iron parameters and haptoglobin levels.

The following day, at the end of 5 weeks, pigs were euthanized by electrical stunning followed by exsanguination. The alimentary canal was removed, and 5 cm segments of the jejunum, ileum, cecum, as well as proximal, mid, and distal colon were excised and cut longitudinally to open. Segments were then transferred to individual tubes containing 25 mL of ice-cold brain heart infusion broth (BHIB) containing 20% glycerol. Luminal microbial populations were recovered by gentle agitation of tubes on ice for 10 min, similar to a method that has been previously described (*25*). Intestinal washes were stored at −80 °C until microbial analyses were performed using terminal restriction fragment length polymorphism (TRFLP).

Experiment 2: Direct Cecal Infusion Study. Nine iron-deficient, 4-week-old male weaner piglets (Yorkshire × Hampshire × Duroc) were randomly selected from litters at the Cornell University Swine Farm. Animals were housed and cared for in the same manner as described for experiment 1, with the following exceptions: all pigs were administered the inulin diet for the 6 week duration of the study (no basal diet group); the composition of the inulin diet remained the same, but we prepared the vitamin and mineral mix ourselves rather than purchasing a premixed formulation. Feed and water intake were monitored daily; feces was scored daily (*24*); and animal weights were determined weekly.

In week 4, pigs were administered a 1 time oral dose of 10 mg of ^{54}Fe (99.84% enrichment; Isoflex, CA), solubilized in 0.01 mol/L HCl and ascorbic acid (AA) at a molar ratio of 5:1 (AA/^{54}Fe), mixed in the morning feed. The following day, pigs were administered a 1 time cecal infusion of 6 mg of ^{58}Fe (92.8% enrichment; Isoflex, CA), solubilized in 0.01 mol/L HCl and AA at a molar ratio of 5:1 (AA/^{58}Fe). Because of time limitations in the surgery room, we could only process 3 pigs per day (Monday, Wednesday, and Friday); therefore, the dosing schedule had to be staggered accordingly. Ascorbic acid was added to ensure that the iron isotope remained soluble in the dosing solutions to ensure accurate delivery of the isotopes. Because only a single isotope dose was administered in this study compared to the seven administered consecutively in experiment 1, doses were increased accordingly. The oral and cecal doses were designed to measure total and colonic iron absorption, respectively. Small intestinal iron absorption was calculated by subtracting the large intestinal absorption from that of the whole intestine.

To perform the cecal infusions, anesthesia was induced with intramuscular administration of Midazolam (0.2 mg/kg) and Ketamine (5 mg/kg) and maintained with sevoflurane. The apex of the cecum was externalized through a 10 cm abdominal incision for infusion using a needle and syringe. Upon infusion, the contents of the cecum were gently agitated to ensure mixing, after which the infusion site was sealed with a purse string suture, the cecum was returned to the abdomen, and the incision site was sutured closed. All pigs received a pre- and post-operative dose of analgesic (Flunixin meglumine, 1.1 mg/kg) intravenously (IV) and antibiotic (Ceftiofur Sodium, 5 mg/kg) IV. No further treatments were required because all surgeries proceeded without complication and all piglets recovered well and exhibited normal activity and feed/water intake within 24 h post-operatively.

Whole blood was drawn from the anterior vena cava prior to oral and cecal stable isotope dosing and 14 days thereafter for hemoglobin and stable isotope ratio analyses.

Blood Analysis. Hemoglobin (Hb) concentrations were determined spectrophotometrically using the cyanomethemoglobin method (Pointe Scientific, Canton, MI). Total body hemoglobin Fe was calculated for each pig at the initial and final blood draw from hemoglobin levels and body weight (BW) using the following formula:

$$\text{Hb Fe(mg)} = [\text{BW(kg)}][0.081\ (\text{L blood/kg BW})][\text{Hb(g/L blood)}][3.35\ (\text{mg Fe/g Hb})]$$

where a blood volume equal to 8.1% BW was assumed (*26*). From these values, HRE was determined using the following formula (*27*):

$$\text{HRE} = \frac{\text{Hb Fe (mg) (final)} - \text{Hb Fe (mg) (initial)}}{\text{total Fe intake (mg)}} \times 100$$

Inductively coupled plasma mass spectrometry (ICP−MS) with an Agilent 7500CS analyzer mass spectrometer was used to analyze the amount of Fe stable isotope (^{58}Fe or ^{54}Fe) in excess of the naturally occurring amount in the total circulating Hb of the animals, thereby providing a measure of Fe absorption (*28*).

Incorporation of a stable isotope tracer into Hb was calculated using a previously published formula (*29*). The percent of ^{58}Fe and ^{54}Fe that was absorbed from the administered dose was then calculated, assuming a 90% incorporation of absorbed Fe into Hb (*30*).

Serum haptoglobin levels were determined spectrophotometrically (Pig haptoglobin ELISA test kit, Life Diagnostics, PA) according to the instructions of the manufacturer. Serum iron, total iron binding capacity, and transferrin saturation were also determined spectrophotometrically (iron TIBC reagent, Raichem, CA).

TRFLP. Microbial community DNA was extracted from intestinal wash samples with the QIAamp DNA Stool Mini Kit (Qiagen, CA) according to the instructions of the manufacturer. The bacterial 16S rRNA gene was amplified with the primers 27f-1492r (*30*), with the 27f primer labeled with 6-carboxyfluorescein (6-FAM, Integrated DNA Technologies). PCR reactions were performed using 10 ng of community DNA template in a 50 μL reaction mix containing 25 μL of GoTaq Green Master Mix (Promega) and 15 pmol of each primer. The thermocycling conditions were similar to those described by Kennedy and colleagues (*31*); however, in this case, an annealing temperature of 52 °C was used. PCR amplicons were purified using the Wizard SV gel and PCR cleanup system (Promega), after which 500 ng of PCR product was digested with 1 U of *Bsh*1236I (Fermentas) for 2 h at 37 °C. Digested DNA was recovered by ethanol precipitation and resuspended in HIDI formamide (Applied Biosystems), to which 0.2 μL of LIZ 600 bp size standard (Applied Biosystems) was added. Terminal fragments were detected and sized on an ABI 3730 DNA sequencer (Applied Biosystems). Raw data were analyzed with Peak Scanner Software (version 1.0, Applied Biosystems). The relative abundance of each phylotype (peak) within the community was calculated according to the method described by Jernberg and colleagues (*32*), and these values were used for an analysis of phylotype abundance differences between the two treatment groups. Putative phylotype identifications were performed by ribosomal database mining (http://www.cme.msu.edu/RDP) (*32*, *33*).

Statistical Analysis. All data are presented as means ± standard error of the mean (SEM) and were analyzed by one-way analysis of variance (MINITAB Release 14.20, State College, PA). Means were considered significantly different at $p < 0.05$.

RESULTS

Experiment 1: Cannulation Study. *Health Status of Pigs*. To ensure that the surgical implantation of the cannulas or the experimental diets did not adversely affect health, pigs were continuously monitored and scored for ill effects based on activity level, water intake, feed consumption, fecal consistency, and weight change. Suture sites and cannulae were also monitored closely for signs of blockage, irritation, and/or infection. All pigs remained active, and no feed or water refusals were observed throughout the duration of the study. All surgeries proceeded without complication. All pigs recovered well and exhibited normal activity and feed/water intake within 24 h post-operatively. The mean fecal score of control pigs and inulin-supplemented pigs was 2.1 ± 0.4 and 1.7 ± 0.3, respectively ($p > 0.05$). Despite a slight lag in growth rates during the surgical period, all pigs showed a steady increase in weight over the 5 week experimental period from an initial weight of about 6 kg to a final weight of about 12 kg. As expected, weights of the cannulated pigs were lower than the benchmark for healthy pigs of similar age (*34*, *35*), most likely because of the stress of the surgery.

Serum Haptoglobin. As a secondary measure of pig health, haptoglobin levels were measured in serum collected during the final blood draw. Haptoglobin is an acute phase protein that is elevated in pig serum as a result of inflammation and infection. The mean haptoglobin concentration was 1.1 ± 0.1 and 1.0 ± 0.1 mg/mL for control and inulin-supplemented pigs, respectively ($p > 0.05$). This corresponds with the value of 1 mg/mL reported for healthy pigs (*36*).

HRE. Consistent with an iron-deficient state, the initial hemoglobin concentrations in blood were 8.4 ± 0.2 and 8.1 ± 0.4 g/dL for control and inulin-fed pigs, respectively. Hemoglobin values rose to a final level of 10.6 ± 0.6 g/dL in controls and 10.2 ± 0.7 g/dL in inulin-supplemented pigs (no statistically significant difference). No significant difference was observed in HRE values between the two treatment groups, which were calculated to be 24.9 ± 4.2 and 22.2 ± 5.3% for control and inulin-fed pigs, respectively.

Serum Iron Parameters. Serum iron levels in control and inulin-supplemented pigs were 95.8 ± 22.8 and 103.0 ± 25.0 μg/dL, respectively. The total iron binding capacity (TIBC) was 402.8 ± 11.3 and 433.8 ± 34.4 μg/dL for control and inulin-fed pigs, respectively. Transferrin saturation was 23.4 ± 5.3 and 22.7 ± 4.0%, respectively. No statistically significant differences were noted between inulin-supplemented and control animals.

Small and Large Intestinal Iron Absorption. **Table 2** summarizes the percentage absorption of the orally administered ^{54}Fe and the cecally infused ^{58}Fe, designed to measure whole and large intestinal iron absorption, respectively. Small intestinal iron absorption was the calculated difference between total and large intestinal absorption. Iron absorption in the small intestine of inulin-supplemented pigs was slightly higher than that of the controls; however, this did not reach statistical significance. Iron absorption in the colon was consistently low in all animals. Small intestinal iron absorption was not significantly different from the whole intestinal iron absorption in either treatment group ($p = 0.971$ and 0.886 for inulin and controls, respectively). In contrast, there was a significant ($p < 0.05$) difference between small and large intestinal iron absorption in both groups.

Enteric Microbiota. **Figures 1** and **2** depict the microbial community profiles obtained for the different intestinal regions of inulin-supplemented and control pigs following *Bsh*1236I digestion. It is immediately apparent that inulin supplementation modulated some microbial populations in both the small and large intestine, although the overall community structure was not altered too dramatically. While a visual comparison of profiles such as this is useful to provide initial information on the populations present in the community and how they have been affected by the treatment regime, it is important to recognize that between animal variations in the ratio of microbial DNA to other DNA in the original intestinal samples can lead to differences in the observed fluorescence intensity on TRFLP profiles. To eliminate this effect, raw peak (hereafter referred to as phylotype) area values should always be converted to a percentage abundance value before performing any statistical or other analyses on microbial community data. To facilitate phylotype comparisons, phylotypes were ranked according to their abundance and the top 30 phylotypes were arbitrarily designated as being dominant

Table 2. Experiment 1: Comparative Iron Absorption Level in Different Intestinal Compartments of Anemic Pigs Administered a Basal (Control) Diet or a Diet Supplemented with 4% Inulin[a]

iron absorption (%)[b]	control	inulin	*p* value
whole intestine (Fe^{54})	20.7 ± 3.5	23.2 ± 2.7	0.810
large intestine (Fe^{58})	1.0 ± 0.2	0.4 ± 0.1	0.298
small intestine	19.7 ± 3.5	22.8 ± 2.7	0.760

[a] Means ± SEM, $n = 5$ per treatment group. [b] Absorption in the whole intestine was distinguished from that of the large intestine by administering Fe^{54} orally and Fe^{58} infused into the cecum via the cannula. Small intestinal absorption was the calculated difference between the whole and large intestinal absorption.

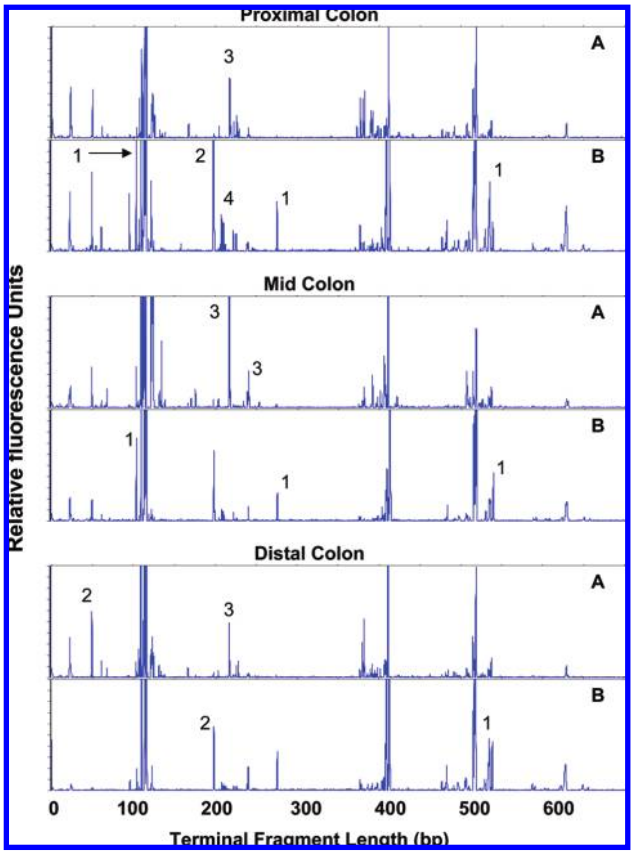

Figure 1. TRFLP analysis of *Bsh*1236I-digested 16S rRNA genes from intestinal washings isolated from the proximal, mid, and distal colon of anemic pigs administered (A) a basal (control) diet or (B) a diet supplemented with 4% inulin. Phylotypes that differed between the two treatments are indicated: 1, *Lactobacillus* spp.; 2, identity unknown; 3, *Clostridium* spp.; 4, *Bifidobacterium* spp.

phylotypes, while all others were classified as minority members of the community. Tables summarizing this data can be found in the Supporting Information.

In the distal colon, there was a 411% increase in the abundance of phylotype 207 (P207) corresponding to *Bifidobacterium* spp. as well as a 38% increase in P227 also corresponding to *Bifidobacterium* spp. The abundance of P538 (*Lactobacillus* spp.) was increased by 950%, causing this phylotype to move from a minority to a dominant classification, while P409 (*Lactobacillus*/*Paenibacillus*/*Eubacterium* spp.) was also increased by 278%, although this population was dominant in both control and inulin pigs. Other changes in the community included a significant reduction from dominant to minority status in phylotypes corresponding to *Clostridium* spp. (P60 and P131; $p < 0.05$), as well as a significant 84% reduction in P216 ($p < 0.05$), which could not be assigned an identity using the ribosomal database and may

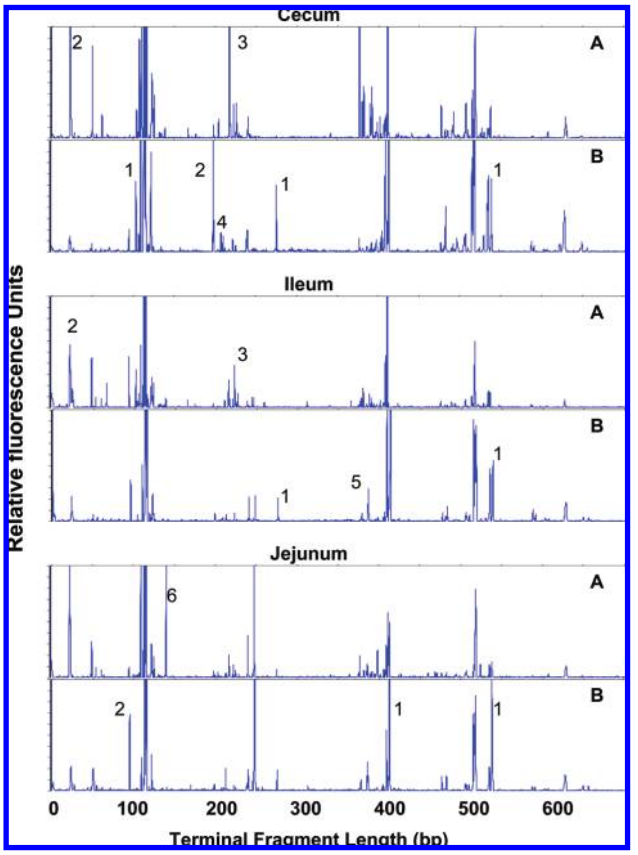

Figure 2. TRFLP analysis of *Bsh*1236I-digested 16S rRNA genes from intestinal washings isolated from the cecum, ileum, and jejunum of anemic pigs administered (A) a basal (control) diet or (B) a diet supplemented with 4% inulin. Phylotypes that differed between the two treatments are indicated: 1, *Lactobacillus* spp.; 2, identity unknown; 3, *Clostridium* spp.; 4, *Bifidobacterium* spp.; 5, *Bacillus*/*Brevibacillus* spp.; 6, *Eubacterium* spp.

represent a novel species, although it is also likely that its sequence is just not represented in the database. A similar trend in microbial community dynamics was observed in the mid and proximal colon, as well as the cecum, of inulin-fed pigs compared to controls.

Although bifidobacteria were not present in the ileum of control or inulin pigs at appreciable levels, a prebiotic effect of inulin was still observed through a significant increase in *Lactobacillus* spp. (P538; $p < 0.05$), which shifted this population from a minority to dominant classification. A significant increase in P386 (*Bacillus*/*Brevibacillus* spp.; $p < 0.05$) was observed at the same time as a significant decrease in P406 (*Bacillus*/*Paenibacillus* spp.; $p < 0.05$). Phylotypes corresponding to clostridia, Enterobacteriaceae, enterococci, and streptococci were also decreased. A prebiotic effect was also noted in the jejunum with increases in lactobacilli (P538, P534, and P412) from minority to dominant status, although these did not reach significance.

Experiment 2: Direct Cecal Infusion Study. *Health Status of Pigs.* To ensure that the inulin diet and the surgical infusion of iron into the cecum did not adversely affect health, pigs were continuously monitored and scored for ill effects based on activity level, water intake, feed consumption, fecal consistency, and weight change. Suture sites were also monitored closely for signs of irritation and/or infection. All pigs remained active, and no feed or water refusals were observed throughout the duration of the study. All surgeries proceeded without complication. All pigs recovered well and exhibited normal activity and feed/water intake within 24 h post-operatively. The mean fecal score of pigs averaged over the study period was 1.1 ± 0.04. All pigs showed

a consistent increase in weight over the 6 week experimental period, although gain was slightly below that expected. The stress of the surgery is the most likely explanation for this.

Hemoglobin. Consistent with an iron-deficient state, the initial hemoglobin concentration in blood was 8.5 ± 0.2 g/dL, rising to a final level of 10.2 ± 0.6 g/dL at the end of the experiment.

Small and Large Intestinal Iron Absorption. **Table 3** summarizes the percentage absorption of the orally administered ^{54}Fe and the cecally infused ^{58}Fe, designed to measure whole and large intestinal iron absorption, respectively. Small intestinal iron absorption was the calculated difference between whole and large intestinal absorption. Similar to results obtained in experiment 1, absorption of the cecally infused iron was consistently low in all pigs, averaging 0.4 ± 0.2%. In contrast, small intestinal iron absorption was significantly higher than this at 27.3 ± 4.1% ($p < 0.001$). Small intestinal iron absorption was not significantly different to the whole intestinal iron absorption ($p = 0.946$).

Table 3. Experiment 2: Comparative Iron Absorption Level in Different Intestinal Compartments of Anemic Pigs Administered a Diet Supplemented with 4% Inulin

intestinal segment	iron absorption (%)[a,b]
whole intestine (Fe^{54})	27.7 ± 4.1 a
large intestine (Fe^{58})	0.4 ± 0.2 b
small intestine	27.3 ± 4.1 a

[a] Means ± SEM, $n = 9$. [b] Absorption in the whole intestine was distinguished from that of the large intestine by administering Fe^{54} orally and Fe^{58} infused directly into the cecum. Small intestinal absorption was the calculated difference between the whole and large intestinal absorption. Absorption values bearing the same letter were not significantly different at the 5% significance level.

DISCUSSION

It has been suggested that prebiotics may enhance the bioavailability of intrinsic dietary iron. While the exact mechanisms involved remain to be fully elucidated, microbial fermentation of these nondigestible carbohydrates in the large intestine is believed to play a significant role (*3*). In a recent study using the porcine model, Yasuda and colleagues (*11*) were able to show that inulin supplementation significantly increased iron bioavailability from a corn−soybean meal diet. In this case, iron bioavailability was assessed on the basis of hemoglobin repletion efficiencies, which provide a measure of the total increase in hemoglobin iron against the total iron intake (*27*, *37*). While this approach is extremely useful for demonstrating an effect on total iron absorption, it does not provide more specific information on which intestinal compartments are being affected. It is generally assumed that iron absorption occurs predominantly in the duodenum and that minimal absorption occurs elsewhere in the intestine; however, because prebiotics are believed to function primarily by affecting the growth of selected bacterial populations inhabiting the large intestine, we hypothesized that the inulin-enhancing effect on iron bioavailability also occurs in this region.

In experiment 1, we used a porcine model to compare the contribution of the small and large intestine to total iron absorption following the consumption of corn−soybean meal diets with or without supplemental inulin. In previous studies in our laboratory (*11*), we showed a significant enhancing effect of inulin on iron absorption using 8 pigs per treatment group. However, because of the complexity and costly nature of the surgeries in experiment 1, we were limited to only 5 pigs per treatment group. The use of the stable isotope protocol adopted for this study allowed each animal to act as their own control, and power calculations using previous data sets suggested that this would enable us to detect a difference in iron absorption between our two treatment groups using only 5 pigs per group. However, contrary to previous non-surgical pig studies, between animal variations in experiment 1 were relatively high and this was attributed to the stress of the surgeries and the invasiveness of the cannulation procedure. Thus, the difference in iron absorption between inulin-supplemented pigs and controls did not reach statistical significance. Iron absorption in the large intestine was consistently low in all pigs, and no significant differences were noted for hemoglobin, hemoglobin repletion efficiency, or serum iron parameters, including serum iron, total iron binding capacity, and transferrin saturation. At the onset of the experiment, all pigs had an average hemoglobin level slightly higher than the borderline for severe anemia, which is generally reported as being 8 g/dL (*37*, *38*). Hemoglobin concentrations rose throughout the experiment, reaching a final level just below the cutoff point for anemia in children under 5 of 11 g/dL (*39*, *40*). Cut-off values for anemia for pigs are not clearly defined, but Rincker (*38*) reported that hemoglobin concentrations below 10.7 g/dL could be increased by iron supplementation of the diet, suggesting that young pigs are similar to humans in this regard. Thus, while experimental animals were certainly iron-deficient, they were not severely anemic based on these criteria. It may be that a more significant effect of inulin on iron bioavailability would have been observed had the pigs been more severely anemic at the onset of the study. At the same time however, a more severe anemia would have increased the likelihood of post-surgical morbidity and mortality.

To confirm the result of experiment 1 that iron absorption in the large intestine is negligible in anemic pigs, we performed an additional study using a less invasive means of delivering iron to the cecum. In this experiment, because we were limited to no more than nine animals, we chose to administer all pigs the same inulin-supplemented diet. We reasoned that, if inulin does promote iron absorption in the large intestine, then feeding all pigs an inulin-supplemented diet should increase the likelihood of detecting a significant contribution of colonic absorption to total iron absorption. On the other hand, if inulin has no such enhancing effect, then colonic iron absorption should not make a significant contribution to the total iron absorption compared to that of the small intestine. We were able to confirm with experiment 2 that the colon does not make a significant contribution to overall iron absorption in iron-deficient pigs, under the conditions used in our studies. This result is in agreement with other published studies in pigs and rats (*41*, *42*).

We were able to show that inulin supplementation did promote a favorable microbiota in the intestinal lumens of the pigs. This included increases in beneficial bifidobacteria and lactobacilli, as well as decreases in less desirable populations, such as clostridia and Enterobacteriaceae, which can harbor pathogenic representatives. Of particular interest was the finding that microbial populations in the jejunum and ileum were also affected by dietary inulin supplementation. While bifidobacterial numbers were relatively low in these regions in all pigs, a significant enhancement of lactobacilli was observed in both small intestinal compartments of inulin-supplemented pigs compared to controls, along with reductions in clostridia, Enterobacteriaceae, and enterococci. Prebiotics, such as inulin, are believed to have negligible effects in the small intestine because they purportedly bypass this region undigested and are fermented in the large intestine, where they enhance intestinal health through the promotion of a favorable microbial balance. However, our results suggest that inulin is capable of exerting a significant impact on the small intestine, evidenced here by the positive modulation of microbial populations in the jejunum and ileum of inulin-supplemented pigs. This could be a result of inulin being fermented in

these regions by the resident microbiota, or it may be that the presence of this nondigestible carbohydrate alters the small intestinal environment in ways that impact the growth of the resident microflora (*43*). Changes in the commensal flora in this region can be of particular importance because they can have a profound effect on intestinal barrier integrity and therefore host digestive and absorptive processes (*44*, *45*). In addition, inulin may affect the expression of genes that code for nutrient transporters in enterocytes, and this could also affect absorption. We have shown that relative DMT-1 mRNA concentrations in duodenal and colon tissues were elevated in pigs fed diets supplemented with inulin compared to control pigs (*46*) . Thus, the inulin effect on iron absorption in pigs may be mediated in the small intestine rather than the large intestine.

Generally, pigs are considered to be a good non-primate model for human nutritional and microbiological studies because their nutritional requirements are strikingly similar to humans, they have a similar enteric microbiota, and the physiology of digestion and associated metabolic processes are very similar to that of humans (*47*, *48*). However, it may be that humans and pigs respond differently to prebiotics. Studies in ileostomy patients have shown that prebiotics pass through the small intestine at least 85% intact (*49*, *50*), but there is some conjecture as to whether this holds true in pigs (*51*, *52*). Current research is underway in our laboratory to try and resolve this issue through examining in further detail the interactions between inulin, iron absorption, and the enteric microbiota.

In summary, the prebiotic action of inulin was confirmed by increases in lactobacilli and bifidobacteria with concomitant reductions in clostridia in the large intestine of inulin-supplemented pigs compared to controls. A similar prebiotic effect was also observed in the ileum and jejunum. Colonic iron absorption levels were consistently low in all pigs, suggesting that the colon does not make a significant contribution to total iron absorption in iron-deficient pigs and that inulin does not promote iron absorption in the colon.

Supporting Information Available: Tables 3−6 summarizing the major differences in phylotype abundance observed in the distal colon, cecum, ileum, and jejunum of inulin-supplemented pigs compared to controls. This material is available free of charge via the Internet at http://pubs.acs.org.

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