EFFECTS OF INULIN ON IRON UTILIZATION BY YOUNG ANEMIC PIGS AND IMPLICATIONS FOR HUMAN NUTRITION

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by

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ABSTRACT

Iron deficiency is the most common nutritional disorder in humans affecting more than 2 billion people around the world. Positive effects of supplemental inulin on iron utilization have been reported in anemic rats. Inulin is a non-digestible carbohydrate that is found in various types of fruits and vegetables. Because pigs are better models for humans than rats, we initially conducted two trials to determine the effects of inulin supplementation on the bioavailability of iron in corn and soybean meal to young anemic pigs. Interestingly, supplemental 4% inulin improved utilization of iron intrinsically present in corn and soybean meal by young pigs as evidenced by the increase in blood hemoglobin concentration. This positive effect of inulin was associated with decreased concentrations of sulfide and increased concentrations of soluble iron in the colon digesta, but not with digesta pH or phytase activity.

Two groups have reported > 90% of pre-caecal digestibility of inulin in pigs, and argued against pigs as a proper animal model for humans in this regard. Therefore, we conducted two experiments and collected the digesta samples from various segments of the gastrointestinal (GI) tract (stomach, upper and lower jejunum, ileum, cecum, and three segments of colon) to characterize the hydrolysis profile of inulin. Our data suggested that the cecum is the major degradation site of ingested inulin in the GI tracts of young pigs. Our finding supported continued utilization of pigs as a model of humans for inulin studies.

Because various forms and amounts of inulin are found in fruits and vegetables, two more experiments were conducted to compare the efficacy of different types (short, long and 50:50 mixture of short and long) of inulin on the bioavailability

of iron to young anemic pigs. Although all three types of inulin enhanced hemoglobin concentration, only the supplementation of Synergy 1, a 50:50 mixture of short and long chain inulin, improved hemoglobin repletion efficiency. Moreover, pigs fed the oligofructose (short chain inulin) supplemented diet showed lowered ($P > 0.05$) hemoglobin repletion efficiency compared to the basal diet (BD) or the Synergy 1 fed pigs. Our finding suggested that different chain-length inulin may improve hemoglobin concentrations in pigs through different mechanisms. In summary, supplemental inulin improved dietary iron utilization by young anemic pigs, indicating the potential of enhancing inulin content in staple crops for reducing iron deficiency in humans.

BIOLOGICAL SKETCH

Koji Yasuda was born in Tokyo, Japan on June, 16th of 1982, where he spent his first fifteen years. Koji came to the U.S. to attend boarding school at Kimball Union Academy (KUA) in Meriden, NH. At KUA, Koji played varsity soccer, lacrosse and enjoyed snowboarding. Immediately following his graduation, he entered Union College in Schenectady, NY majored in Biology where he joined the Sigma Chi Fraternity. After two years at Union College, Koji transferred to the College of Agriculture and Life Sciences at Cornell University where he majored in Animal Science. Koji began working as a research assistant in Dr. Xingen Lei's lab during the summer of his senior year of college. Koji completed his Bachelors of Science degree with distinction in research in May, 2005. Koji remained at Dr. Xingen Lei's lab to continue on the project as a masters student. After completing his Masters of Science degree in August of 2007, Koji will begin his study for Doctor of Veterinary Medicine degree at Cornell University's College of Veterinary Medicine.

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BD	Basal Diet
FOS	Fructooligosaccharides
Sc-FOS	Short-chain fructooligosaccharide (synthetic)
HP	High performance inulin, long-chain inulin (DP_{av} = 25)
P95	Oligofructose (DP_{av} = 4); short-chicory inulin
Synergy 1	50:50 mixture of P95 and HP (DP_{av} = 12)
Hb	Hemoglobin
HRE	Hemoglobin Repletion Efficiency
SCFA	Short-chain fatty acids (acetate, propionate and butyrate)
Extpt	Experiment

CHAPTER ONE

INTRODUCTION

1.1 Health and Nutrition

According to the new definition proposed by the World Health Organization (WHO), "health" is no longer limited to the "absence of disease"; it also includes physical and psychological well-being (1). Along with the growing cost of medical care, increased life expectancy, and changes in lifestyles, nutritional health will play a more important role in the future.

Because gut microbes can affect host health, there has been tremendous interest in manipulating the composition of the gut flora towards a potentially "health friendly" community. Attempts have been made to increase bacterial groups such as Bifidobacteria and Lactobacillus, which may have health-promoting properties (2). Initially, "probiotics," defined as microbial food supplements that beneficially affect the host by improving its intestinal microbial balance were advocated. However, changes accomplished by the supplementation of probiotics may be transitory, and the implantation of exogenous bacteria therefore is inadequate.

A newer concept of prebiotics was introduced in 1995 by Gibson and Roberfroid (2) to improve host health. Prebiotics are defined as nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of a limited number of bacterial species already resident in the colon. Nondigestible oligosaccharides in general, inulin and fructooligosaccharides in particular, are prebiotics (2). When these compounds are ingested by humans and animals, modulation of the colonic microbiota occurs though stimulation of growth of endogenous bifidobacteria and lactobacillus. This in turn changes the composition of

the microbiota (3). Moreover, prebiotics have been documented to enhance mineral bioavailability (4-7), modulate lipid metabolism (8-11), and strengthen intestinal immunity (12-15). Health benefits promoted by the supplementation of inulin-type fructans stimulate further understanding of such compounds, as they may help meet the demanding future of nutrition and health.

1.2 Nature of Inulin

Inulin (Fig. 1.1) is a fructan that can be found in plants. It functions as an osmotic-regulator and is a storage form of carbohydrates (16). From a chemical point of view, the linear chain of inulin is either a ß-$_D$-glucopyranosyl-[- ß-$_D$-fructofuranosyl]$_{n1}$- ß-$_D$-fructofuranoside($G_{py}F_n$) or a ß-$_D$-fructopyranosyl-[- ß-$_D$-fructofuranosyl]$_{n1}$- ß-$_D$-fructofuranoside ($F_{py}F_n$). In both cases, the fructosyl-glucose and fructosyl-fructose linkages have a ß2→1 bond at the anomeric C_2 (17). Inulin is mainly found in plants, but it is also present in fungi and bacteria. In plant fructans, the number of fructose monomers does not exceed 200, whereas in bacterial fructans, it can be as high as 100,000 and is highly branched (18). Inulin also has been documented to exist in a cyclic form that contains 6, 7, or 8 fructofuranose rings (16). Distribution of inulin in plants has been analyzed by Van Loo et al, (19). Table 1.1 was directly taken from that publication, and is a summary of inulin content and chain length of miscellaneous plants, which illustrates the diversity of inulin types in different plant species. Inulin content ranges from less than 1 up to some 20% of fresh weight (Chicory). Moreover, not considering the structure of inulin (linear or linear and branched), the length of the chain also varies. Based on these data, the average daily consumption of the various types of inulin has been estimated to be between 3

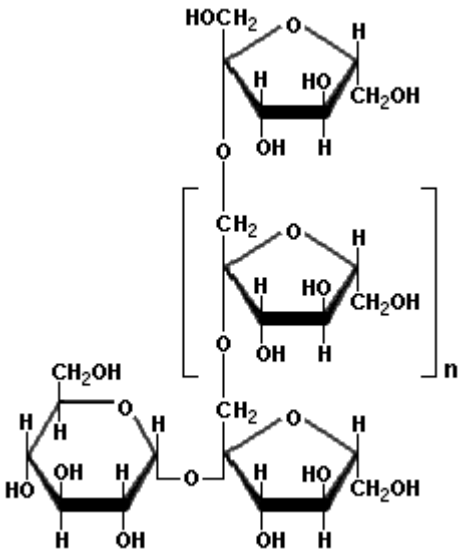

Figure 1.1 The chemical structure of inulin. Picture depicts ß-$_D$-glucopyranosyl-[ß-$_D$-fructofuranosyl]$_{n1}$- ß-$_D$-fructofuranoside($G_{py}F_n$).

and 11 g in Europe (19) and between 1 and 4 g in the U.S (20), the most common sources being, in both studies, wheat, onion, banana, garlic, and leek. Because of its high inulin content (16 – 18%) and consistent recovery of its content from year to year, commercial inulin production is heavily dependent on chicory or Jerusalem artichoke, shows a higher inulin content (17 – 20.5%). The shape of Jerusalem artichokes is highly irregular and a large amounts of soil are attached, making it not useful for inulin production (21).

Chicory inulin is a mixture of oligomers and polymers in which the degree of polymerization (DP) varies from 2 to about 65 units with a $DP_{av} = 12$. About 10% of the fructan chains in native chicory inulin have a DP ranging between 2 (F_2) and 5 (GF_4). Partial enzymatic hydrolysis of inulin with an endoinulinase, produces oligofructose that is a mixture of both $G_{py}F_n$ and $F_{py}F_n$ molecules, in which the DP varies from 2-7 with a $DP_{av} = 4$ (22). Oligofructose can be obtained by enzymatic synthesis (transfructosylation) using the fungal ß-fructosidase from *Aspergillus niger*. In this reaction, in a process similar to the plant biosynthetic pathway, sucrose serves as a substrate to which 1, 2, or 3 additional fructose units are added by forming new ß-(2,1) linkages (23). In such a synthetic compound, DP varies from 2-4 with $DP_{av} = 3.6$ and all oligomers are the $G_{py}F_n$-type. Because the inulin hydrolysate (oligofructose) and the synthetic compound have a slightly different $DP_{av} = 4$ vs. 3.6, the synthetic compound (DP_{av} of 3.6) has been called short-chain fructooligosaccharides (Sc-FOS).

Furthermore, by applying specific separation technologies, the food industry (Orafti, Tienen, Belgium) also produces a long-chain inulin known as inulin HP (DP 10 – 60) with a $DP_{av} = 25$. Finally, a specific product known as Synergy1® is produced, also from Orafti by combining chicory oligofructose and long-chain inulin at a 50:50 ratio (24). This is the type of inulin that was used in our studies, except for those discussed in Chapter 4.

Table 1.1 Inulin content and chain length of miscellaneous plants.

Plant	Inulin (g/100g)	Chain Length Degree of polymerization (DP)
Globe Artichoke (*Cynara scolumus*)	2-7	DP ≥ 5 = 95%
Banana (*Musa cavendishii*)	± 1	DP ≥ 40 = 87%
Barley (*Hordeum vulgare*) very young kernels	0.5 – 1 ± 22	DP < 5 = 100%
Chicory (*Cichorium intybus*)	15 – 20 Mean 16.2	DP < 40 = 83% DP 2 – 65 DP ≥ 40 = 92%
Dandelion (leaves) (*Taraxacum officinale*)	12 – 15	
Garlic (*Allium sativum*)	16 Mean 13	DP ≥ 5 = 75%
Jerusalem Artichoke (*Helianthus tuberosus*)	17 – 20.5	DP < 40 = 94% DP 2 – 50 DP ≥ 40 = 6%
Leek (*Allium ampeloprasum*)	3 – 10	DP 12 is most frequent
Onion (*Allium cepa*)	1 – 7.5 Mean 3.6	DP 2 – 12
Salsify (*Scorzonera hispanica*)	Mean ± 20	DP ≥ 5 = 75%
Wheat (*Triticum aestivum*)	1 – 4	DP ≤ 5 = 50%

1.3 Digestion and Fermentation of Inulin

In the human diet, the most common carbohydrates are dietary fibers, starch, sucrose, lactose, fructose, glucose and, to a lesser extent, inulin and other types of fructans. Most (50-60% of daily intake) carbohydrates are starch, which is a mixture of linear (amylase) and branched (amylopectin) polymers of glucose with α-1, 4 and α-1, 4 + α-1, 6 linkages, respectively (25). Starch and the disaccharides lactose and sucrose are hydrolyzed in the upper part of the GI tract, essentially in the oral cavitity and the small intestine (or at least the hydrolysis process starts at those sites) while dietary fibers and inulin-like fructans are not. The monosaccharides in the diet (glucose and fructose) are produced by the hydrolysis of starch and dissaccarides (lactose and sucrose). These are absorbed and reach the circulation via the portal vein (25). On the other hand, monosaccarides derived from fiber and inulin that reach or are produced in the large intestine (including the cecum in some animals), are essentially not absorbed, but are fermented.

It is well documented that inulin-type fructans are resistant to stomach acid, and enzymatic (saliva, pancreatic, and small intestinal) hydrolysis in humans (26-29). While these enzymes are specific to α(1→4) or α(1→6) linkages, inulin and oligofructose have almost exclusively ß(1→2) (and a few (6→2) linkages. In pigs, however, the site of inulin degradation in the GI tract is a subject of debate. While currently available data suggests that inulin and oligosaccharides are digested (90-100%) before reaching the colon (30,31), these pig studies had major technical limitations preventing from precisely identifying the location of inulin degradation. Inulin disappearance was based on digesta samples collected from the anus of ileostomy patient-mimicked pigs that underwent a surgical procedure to connect the terminal ileum to the proximal section of 20-25 cm of intact rectum.Evidently

neglecting the hydrolysis of inulin in the rectum (30). The other investigator simply subtracted the basic nutrient components (ash, crude protein, ether extract, and crude fiber) from dry matter to derive inulin content (31). Further studies are needed to identify the site(s) of inulin degradation in pigs using more direct and sensitive methods (see Chapter 3 of this thesis). When undigested inulin-type fructans reach the large intestine, they are readily fermented by a wide variety of bacteria. Carbohydrate fermentation is an important force that drives the ecology and the physiology of the large intestine. Major hydrolytic steps in colon degradation of common nondigestible carbohydrates are shown in Figure 1.2 (21). Following the hydrolytic step, carbohydrates are anaerobically oxidized to pyruvate (Figure 1.3). Pyruvate is then utilized by the microbes, which produce short-chain fatty acid (SCFA) and other compounds (Figure 1.4). Predictably, no single bacteria are capable of producing all the metabolites; in fact most if not all, bacteria in the large intestinal community contribute to complex metabolic interactions.

Both *in vivo* and *in vitro* studies have demonstrated that inulin and oligosaccharide selectively stimulate the growth of bifidobacteria and/or lactobacilli, both of which are considered to be beneficial to the host (32-38). Increased populations of these bacteria have the ability to suppress putrefactive and potentially pathogenic organisms like enterobacteria and certain clostridia (38). In addition, the enhanced production of SCFA, fermentation end products, following feedings of inulin and other types of fructans, have been well documented in human and animals (21,33,39-41). It is believed that a number of the inulin related health benefits are associated with this increase of SCFA production in the large intestine. Butyrate, for instance, plays an essential role in the maintenance of colonic mucosal integrity by acting on the metabolism, proliferation, and differentiation of different epithelial cell types (42). Enhanced proliferation and differentiation of different epithelial cell types

may partly explain the inulin-associated enhancement of mineral bioavailability by butyrate.

1.4 Inulin and Bioavailability of Minerals

The beneficial effects of inulin and oligofructose on mineral bioavailability have been well documented in many different species, including humans (4,5,7,27,43-49). Supplemental dietary inulin has been shown to improve the bioavailability of calcium and magnesium in animals and humans (6,7,49-51) and to a lesser extent Fe (4,5,44,52). Data for the effect on Cu and Zn bioavailability are limited (44,53,54). The effects of inulin-type fructans on iron status are summarized in Table 1.2. Several different sources of inulin (Sc-FOS, FOS, and chicory inulin, but not inulin HP) have been studied in species including rats, pigs and humans, with supplementation levels ranging from 1.5% to 10%. Duration of studies has varies from a10-day rat study to studies lasting for 6 weeks. From the studies listed in table 1.1, Delzenne et al (44), Hubert et al, Ohta et al (5), and Sakai et al (52), showed an enhanced iron absorption (apparent absorption), retention, hemoglobin repletion efficiency (HRE) and hemoglobin and recovery from post-gastorectomized anemia in rats, respectively.

1.5 Iron Uptake and Regulation

Iron is the second most abundant metal in the earth's crust (55). It exists almost exclusively in its oxidized state (Fe^{3+}). The low bioavailable of this form accounts for the widespread prevalence of iron deficiency. Regulation of body iron occurs at the level of absorption. Absorption is tightly associated with the size of body iron stores,

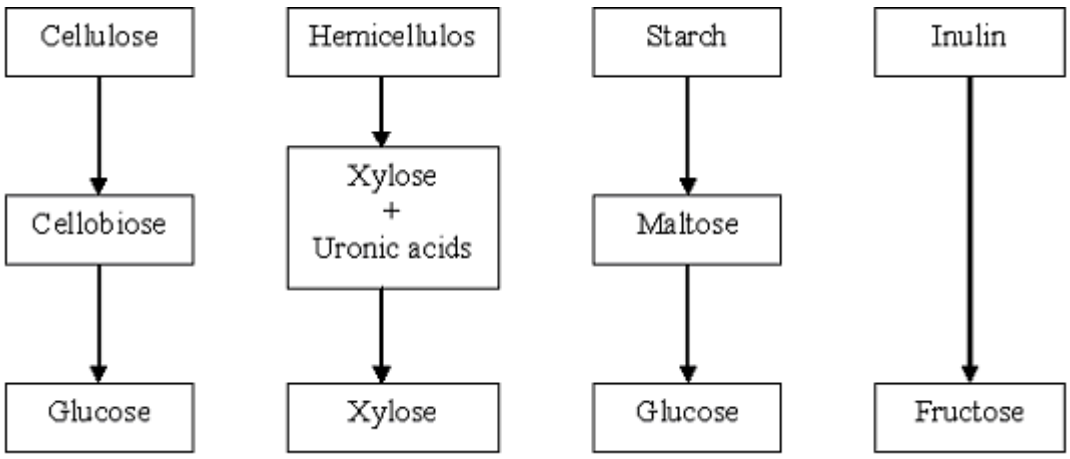

Figure 1.2 Major hydrolytic steps in the colonic degradation of the most common nondigestible carbohydrates (21).

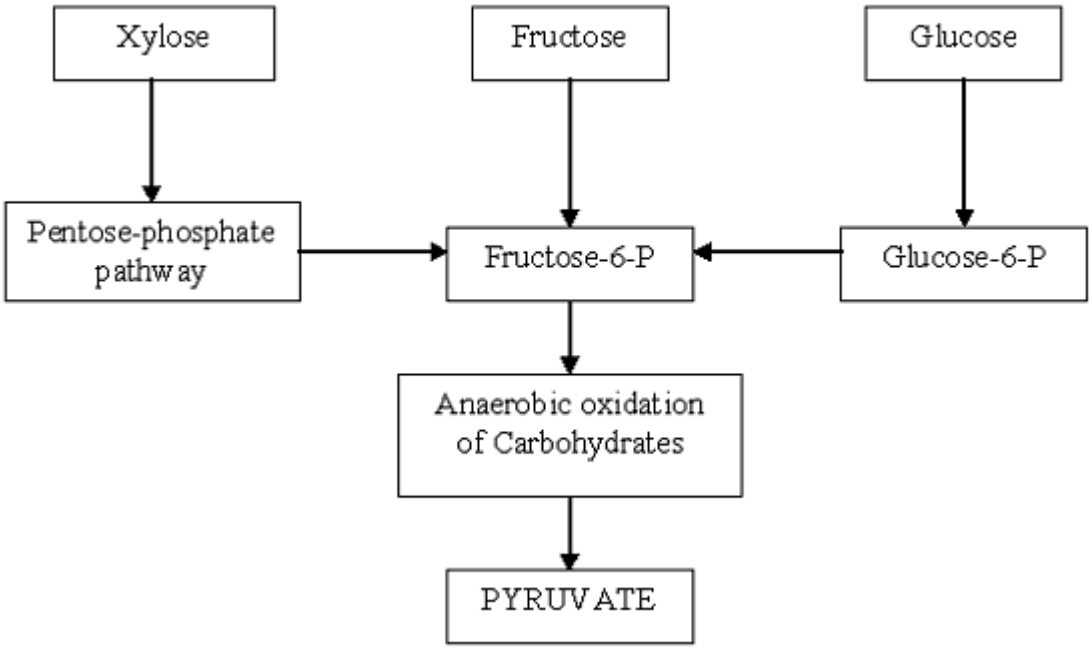

Figure 1.3 Major metabolic pathways in the anaerobic oxidation of carbohydrates to pyruvate (21).

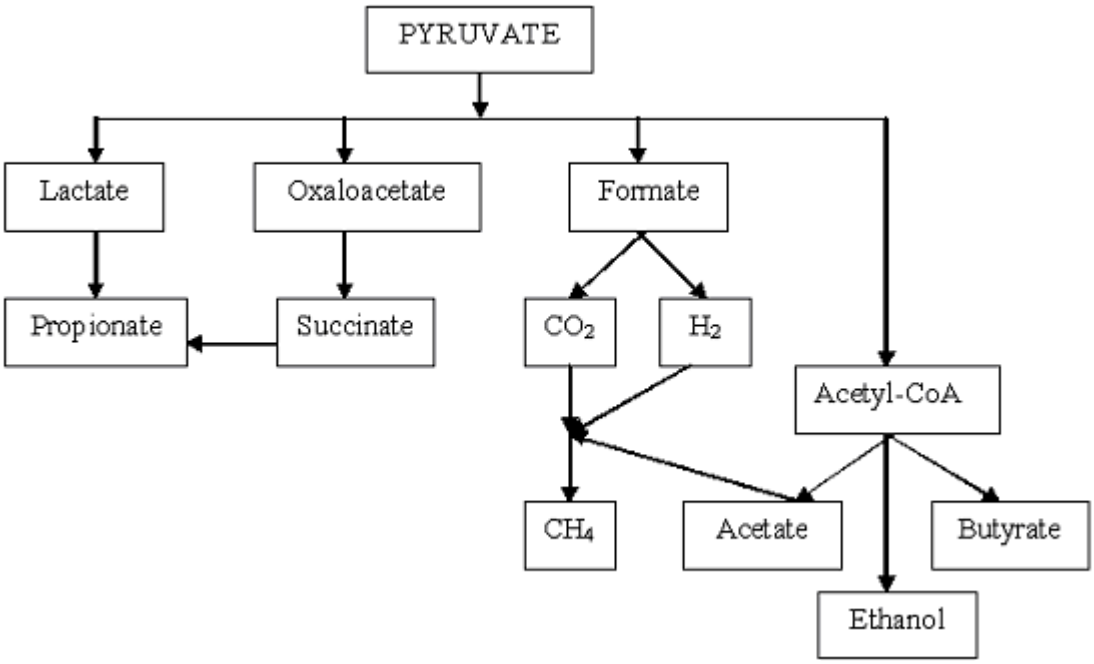

Figure 1.4 Main metabolic pathways that utilize pyruvate in colonic microflora (21).

rising with iron depletion and falling when stores are increased (55). Various factors affect iron bioavailability. At the dietary level, inhibitors include phytate (56), phenols (55) and sulfide and facilitators include ascorbic acid (57), meat products (57) and supplemental phytase (58). In addition, a reduction in pH or an elevation in the level of luminal water content contributes to increased solubility, which in turn can enhance iron bioavailability. At the cellular level, the abundance of the brush border membrane iron transport protein (Divalent metal transporter 1:DMT1), basal lateral transporter protein (ferroportin), cellular iron storage protein (ferritin), plasma iron transport protein (transferrin) and many other proteins have the ability to affect iron bioavailability. To best of our knowledge, no one has investigated the effects of inulin on these parameters. Therefore, it is interesting to explore whether inulin affects regulation of these iron-related proteins.

1.6 Pigs as a Human Model

Due to striking similarities in body composition, metabolism and digestion, pigs have been used as models to study many different human biological processes, including immunology, dermatology, diabetes and nutrition (59). Young pigs are a particularly good model to study human iron nutrition, not only because they have similar anatomy and physiology of the GI tract, but also, because their iron status can be readily manipulated by adjusting the dose of the iron injections that are routinely given shortly after birth due to the low iron level (about 1 ppm) in sow's milk (60). Additionally, the rapid growth rate of the weanling pig (1000% increase of birth weight in the first six weeks,) allows the animals to reach an "iron deficient state" in a relatively short period of time.

Controversy exists on the mechanism of inulin-type fructan degradation between humans and pigs. Humans, when inulin concentrations are compared in feces and effluents of the ileostomy show virtually no degradation (26-29,61) or absorption(62,63) of inulin anterior to the large intestine is observed. In contrast, Branner et al(30) and Houdijk et al (31) have reported > 90% pre-caecal digestibility of inulin inpigs, suggesting that pigs were not a proper animal model for humans (30,31). Because these pig studies have had major technical limitations for precisely identifying the location of inulin degradation (see Chapter 4), we have used a more direct and sensitive methods to clarify these issues.

1.7 Research Objectives

Our first objective was to determine whether and how supplemental inulin improved utilization of iron intrinsically present in a corn and soybean meal diet by young pigs for hemoglobin repletion.

Our second objective was to characterize the site of inulin degradation, profiles of other carbohydrates, and the activity of the inulin degrading enzymes in various segments of the GI tract in young pigs.

Our third objective was to compare the efficacy of different types of inulin-type fructans on the bioavailability of iron in corn and soybean meal to young pigs.

Table 1.2 Summary of the effects of inulin-type fructans on Fe status

Reference	Sources	Levels	Duration	Species	Fe Status
Delzenne et al., 1995	Raftilose® (DP:4.8) Raftiline® (DP:10)	10% (1.6 g/d)	24 days	Rat (100g)	↑ Fe retention
Hubert et al., 2000	Chicory Inulin	10% w/ 0.7% phytic acid	10 days	Rats (160g)	↑ Fe absorption
Ohta et al., 1995	FOS	5%	2 wk	Fe-deficient rats	↑ HRE ↑ Hb ↑ Hematocrit ↑ Cecal soluble Fe
Ohta et al., 1998	FOS	7.5%	6 wk	Gastorectomized anemic rats	↑ Serum Fe ↑ HRE
Yasuda et al., 2006	Synergy1®	2%, 4%	5, 6 wk	Anemic pigs	↑ HRE ↑ Hb
Sakai et al., 2000	(Raftiline®) Sc-FOS (Meioligo®)	7.5%	6 wk	Rat (4wk old); Gastorectomized anemic rats	↑ Hb,HRE by Sc-FOS No effect on inulin
Sakai et al., 1998	Sc-FOS Raftiline® (DP:10)	7.5%	6 wk	Gastorectomized anemic rats	↑ HRE in Sc-FOS fed rats - HRE in inulin fed rats
van den Heuvel et al., 1998	Inulin	15 g/d	3 wk	Man (20-30 yrs old)	No effect

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CHAPTER TWO

Inulin Improved Iron Bioavailability

2.1 Abstract

Iron deficiency represents one of the most common global nutritional disorders in humans. Our objective was to determine whether and how supplemental inulin improved utilization of the iron present in a corn-soybean meal diet by young pigs for hemoglobin repletion. In Experiment 1, three groups (n = 8/group) of animals were fed a corn-soybean meal based diet (BD, without inorganic iron addition) or BD + 2 or 4% inulin (Synergy 1: a mixture of oligofructose and long chain inulin HP, Orafti, Tienen, Belgium) for 5 wk. Final blood hemoglobin concentrations and the overall hemoglobin repletion efficiency of pigs were positively (r = 0.55 and 0.69, $P < 0.01$) correlated with dietary inulin concentrations. Compared with pigs fed BD, those fed 4% inulin demonstrated a 28% improvement ($P < 0.01$) in hemoglobin repletion efficiency and 15% ($P < 0.01$) improvement in the final blood hemoglobin concentrations. In Experiment 2, 12 weanling pigs (n = 6/group) were fed BD or BD + 4% inulin for 6 wk. Pigs fed 4% inulin had higher ($P < 0.05$) soluble Fe concentrations in the digesta of the proximal, mid, and distal colon, and lower ($P < 0.05$) sulfide concentrations in the digesta of the distal colon. Supplemental inulin had virtually no effect on the pH or phytase activity of digesta from any of the tested segments. In conclusion, supplementing 4% inulin improved utilization of intrinsic iron in the corn-soybean meal diet by young pigs, and this benefit was associated with soluble Fe and sulfide concentrations but not pH or phytase activity in the digesta.

2.2 Introduction

Iron deficiency is the most common nutritional disorder in humans affecting >2 billion people around the world (64). Although food fortification and iron supplements have been used to effectively combat this problem in certain regions (65), these interventions are very difficult to sustain and often do not reach the most "at risk" groups. Because staple food crops (e.g., rice, wheat, maize, beans, cassava, and sweet potato) are major dietary sources of iron for people in developing nations, enhancing bioavailable iron in these crops could be the most effective and sustainable strategy to reduce or prevent iron deficiency in these populations (66).

Iron bioavailability of staple crops may be improved by removing or reducing inhibitors of iron utilization (e.g., phytate and polyphenolics) and/or by enriching enhancers of iron utilization (67). Recently, inulin and short-chain fructooligosaccharides (FOS)[5] have been studied as possible candidates for such enhancers (5,44,51,52,68). These compounds are unique D-fructofuranose polymers linked by a β2→1 bond at the anomeric C_2, and they accumulate in the tissues of many plant species (17). The general assumption is that these compounds are indigestible in the upper digestive tact of simple-stomached animals and humans (69), but pass to their lower gut to be fermented by microbes (17).

Positive effects of supplemental inulin or FOS on bioavailability of dietary calcium and magnesium in animals and humans have been reported (53,70-74). However, only a few studies (5,44,48,51,52,68) have been conducted to determine such effects on the bioavailability of dietary iron.

Consequently, several major issues remain to be clarified. First, the effects of inulin or FOS on dietary iron utilization are inconclusive as determined by hemoglobin (Hb) and hematocrit concentrations in rats fed diets containing 5 or 7.5% FOS (5,52). No improvement in iron utilization was observed by supplementation of inulin or FOS

in healthy men (51,68). Second, all experimental animals or test subjects in previous studies were fed inorganic iron supplements (5,48,52). Thus, the exclusive effects of supplemental inulin or FOS on the bioavailability of iron intrinsically present in foods of plant origin have not been studied. Lastly, there is little information on the mode of action of inulin in enhancing iron bioavailability.

Because both i*n vivo* and *in vitro* studies have demonstrated that inulin and FOS stimulated the proliferation of certain types of colonic bacteria, such as *bifidobacteria* and *lactobacilli* (32-34,36,75-78), most inulin studies on mineral nutrition have been focused on the possible changes of these microbial populations (33,34,36,75-78) or the fermentation products of inulin such as short-chain fatty acids in the hindgut (33,34,76). However, effects of inulin on microbial production of two putative iron solubility and(or) bioavailability determinants, hydrogen sulfide and phytase, in the digesta of various segments have not been well studied. Hydrogen sulfide generated from sulfate and sulfur amino acids by gut microbes (79) may react with ferrous iron to form insoluble ferrous sulfide, inhibiting its absorption. In contrast, microbial phytase releases phytate-bound iron from the digesta and renders it available for absorption (58,80,81). In addition, there is little evidence for a direct impact of inulin on iron concentration or solubility in the digesta, particularly in the upper digestive tract.

Young pigs are an excellent model to study human iron nutrition because of similarities in the anatomy of their gastrointestinal tracts, digestive physiology, and diets between the two species (60). The iron status of young pigs can be readily manipulated by adjusting the dosage of the iron injections routinely given shortly after birth. The tremendous growth rate of weanling pigs (10-fold increase of birth weight in the first six weeks of life), the low iron body stores, and the low iron intake from sow's milk (1 mg/L) allows these animals to develop an "iron deficient state" in a relatively short period of time (60). Therefore, the objectives of this study were to determine if supplemental 2 and 4% dietary inulin: 1) improved utilization of iron intrinsically

present in a corn-soybean meal basal diet by young pigs for hemoglobin synthesis; and 2) affected sulfide and soluble Fe concentrations, phytase activity, and pH of digesta from different segments of the gastrointestinal tracts of young pigs.

2.3 Materials and Methods

2.3.1 Basal Diet and Inulin

The basal diet consisted of corn and soybean meal (**Table 1**), and contained adequate concentrations of all nutrients (82) except iron (no inorganic iron was added). The actual concentrations of iron in all experimental diets were analyzed using an inductively coupled argon plasma emission spectrometer (ICAP 61E Trace Analyzer, Thermo Electron Corporation, Waltham, MA) (83). The actual concentrations of inulin in all experimental diets were determined using the method described by Quemer et al (84) (Table 1). Synergy® 1 (Orafti, Tienen, Belgium) was used as the source of inulin replacing corn starch in the BD. This product is a mixture of α-D-glucopyranosyl-(β–D-fructofuranosyl)$_{n-1}$-β-D-fructofuranosides (n = 10 to 60, average of 25), and oligofructose, α-D-fructopyranosyl-(β–D-fructofuranosyl)$_{n-1}$-β-D-fructofuranosides (n = 2 to 7, average of 4).

2.3.2 Experimental Animals and Protocols

Two experiments were conducted with a total of 36 weanling Yorkshire × Hampshire × Landrace crossbred pigs from the Cornell University Swine Farm. Both experiments were approved by the University Institutional Animal Care and Use Committee. All experimental pigs were selected from litters that were injected with only a half of the normal iron dose (50 mg of iron as Fe-dextran) at birth, and were

allocated to treatment groups based on body weight, litter, gender, and hemoglobin concentrations. Pigs were housed individually in pens with concrete floor in a temperature-controlled barn (22 ~ 25^{o}C) with a light:dark cycle of 12:12 h, given free access to feed and water, and checked daily. In Exp. 1, 24 weanling pigs (body weight = 9.23 ± 0.03 kg) were allotted into three groups (n = 8), and were fed the BD, BD + 2% inulin, or BD + 4% inulin for 5 wk. In Exp. 2, 12 weanling pigs (body weight = 7.70 ± 0.19 kg) were allotted into two groups (n = 6), and were fed the BD or BD + 4% inulin for 6 wk. Prior to the beginning of both experiments, all pigs were fed BD for 2 wk to adjust their body iron stores.

2.3.3 Growth Performance and Sample Collection

In both experiments, feed intake of individual pigs was recorded daily and body weight of individual pigs was measured weekly. Blood samples of all individual pigs (fasted overnight for 8 h) were collected weekly from the anterior vena cava using 5-mL heparin syringes to assay for blood Hb and hematocrit. At the end of Exp. 2, all pigs were killed by electrical stunning and exsanguination. Based on a preliminary experiment, pigs were first fasted for 8 h and then were given free access to feed for 10 h prior to slaughter for us to collect comparable and sufficient digesta samples from all designated segments. The digestive tracts were quickly removed from the carcass and separated into various sections for digesta sampling. Digesta samples for the stomach were collected from the entire contents thoroughly mixed using a blender. Digesta samples for different parts of the intestines were collected from a 12-cm segment each, and the excisions were as follows: upper jejunum, 2-m posterior to the pylorus; lower jejunum, 2-m anterior to the ileo-caecal junction; proximal colon, immediately posterior to the ileo-caecal junction; mid colon, equal length up and down the mid transverse

colon; and distal colon, immediately anterior to the rectum. The samples were immediately frozen in liquid nitrogen, and stored in a -20°C freezer. After 48 h, all samples were freeze-dried (20 SRC-X, Virtis Co. Inc., Gardiner, NY) and stored in a -20°C freezer until analysis. All the assayed values were expressed on a dry matter basis, and moisture contents in the fresh digesta samples were calculated from the weight difference before and after freeze drying.

2.3.4 Blood Sample Analyses

Blood Hb concentrations were measured spectrophotometrically using the cyanomethemoglobin method following the manufacturer's instructions (Pointe Scientific, Inc. Canton, MI). Hematocrit values were determined using heparinized microcapillary tubes (Fisher Scientific, Pittsburgh, PA). Hemoglobin repletion efficiency (HRE) was determined using the following formula (85):

HRE = [(final total body Hb Fe, mg – initial total body Hb Fe, mg)/ total Fe intake, mg)] × 100.

and total body Hb iron content was estimated using the following formula (85):

Hb Fe, mg = [body weight (g) × 0.067 mL blood/g BW] × (Hb, g/mL) × (3.35 mg Fe/Hb, g).

2.3.5 Digesta Sample Analyses

Total digesta Fe concentration was measured using the same method used for dietary Fe concentrations. To determine the pH and soluble iron of digesta and fecal samples, 2 g of fresh wet samples were suspended in 18-mL distilled water and mixed on an rotator stirrer for 30 min at room temperature and centrifuged at 3,000 × *g* (GS-6KR Centrifuge, Beckman Instruments Inc., Columbia, MD) for 15 min at 4°C. The pH in the homogenates was determined using a glass electrode (Accumet Tris Compatible Combination Electrode, Model 630, Fisher Scientific, Co.). Soluble iron concentration in the prepared homogenates was measured using a ferrozine assay (86). After 0.1 mL of homogenate was diluted in 0.9 mLof deionized water, 0.1 mL of ferrozine chromogen solution was added for color development. The absorbance was measured at 562 nm using a KC-4 version 2.6 microplate scanning spectrophotometer (BIO-TEK® Instruments, Inc. Winooski, VT). Total soluble sulfide concentration in the fresh digesta was determined as previously described (87-89). Digesta phytase activities were measured using a spin column method as described by Kim and Lei (90) at two pH levels: the actual digesta pH for each segment and the commonly used pH (5.5) for phytase activity assay.

2.3.6 Statistical Analyses

Data were analyzed as a randomized block design using the Proc General Linear Models procedure of SAS (version 6.12, SAS Inst., Inc., Cary, NC). Effects of dietary

inulin on various measures were analyzed using one-way ANOVA with or without time-repeated measurements. Dose-dependent effects of inulin in Exp. 1 were analyzed using Proc Reg procedure of SAS. Each individually-penned pig was used as the experimental unit. The *Boneferroni t*-test was used to compare treatment means, and the significance level was set at $P \leq 0.05$ (91). Values in the text are means ± SEM.

Table 2.1 Composition of the experimental diets

Ingredient		BD	+ 2% inulin	+ 4% inulin
			g/kg	
Corn		627.9	627.9	627.9
Soybean meal, 48% CP		258.3	258.3	258.3
Corn oil		20.0	20.0	20.0
Corn starch		40.0	20.0	0.0
Sodium phosphate		11.1	11.1	11.1
Calcium carbonate		10.7	10.7	10.7
Plasma, spray-dried		10.0	10.0	10.0
Sodium chloride		2.5	2.5	2.5
Vitamin/mineral premix[1]		10.0	10.0	10.0
L-Lysine		2.5	2.5	2.5
DL-Methionine		1.0	1.0	1.0
L-Threonine		1.0	1.0	1.0
Tylan 10		5.0	5.0	5.0
Inulin		0.0	20.0	40.0
Total		1000	1000	1000
Nutritional values				
ME[2], MJ/kg		14.1	13.8	13.5
Crude protein[2], %		18.5	18.1	17.6
Crude fiber[2], %		4.3	4.5	4.7
Fe[3], mg/kg	Exp. 1	74.1	74.1	74.1
	Exp. 2	53.1	-	55.7
Inulin[4], g/kg	Exp. 1	2.0	19.0	42.0
	Exp. 2	0.0	-	32.0

[1]Vitamin and mineral premix provided/kg diet: retinyl palmitate, 1208 μg; ergocalciferol, 5.5 μg: *dl*-α-tocopheryl acetate, 10.72 mg; menadione, 0.5 mg: *d*-biotin, 0.05 mg: choline chloride, 0.5 g; folic acid 0.3 mg; niacin, 15 mg; Ca-D-panthothenate, 10 mg; riboflavin, 3.5 mg: thiamin 1 mg: pyridoxine, 1.5 mg; Cyanocobalamin, 17.5 μg; $CuSO_4 \cdot 5H_2O$, 6 mg; $C_2H_8N_2$2HI, ethylene diamine dihydroiodide, 0.14 mg; MnO, 4 mg; Na_2SeO_3, 0.3 mg; ZnO, 100 mg.

[2]Calculated based on NRC (82).

2.4 Results

2.4.1 Experiment 1

While the three groups of pigs had similar initial blood Hb concentrations at wk 0, final blood Hb concentrations in pigs fed 4% inulin was 15% higher ($P < 0.05$) than that of pigs fed BD (**Table 2**). However, the concentration in pigs fed 2% inulin was not statistically different from pigs fed either BD or 4% inulin. The changes in mean Hb concentrations over the 5 wk period between the treatment groups displayed the same statistical outcome as their final blood Hb concentrations (data not shown). Pigs fed 4% inulin had higher ($P < 0.01$) overall HRE than that of pigs fed BD or 2% inulin (**Fig. 1**). The improvement noted for pigs fed 2% inulin was not statistically different from pigs fed the BD. Responses of final blood Hb concentrations and overall HRE to dietary inulin concentrations were described by the following linear regression equations:

Final Hb concentration = $12.71 + 0.48 \times$ % inulin (($R^2 = 0.30$, $P < 0.01$)

Overall HRE = $22.60 + 2.26 \times$ % inulin ($R^2 = 0.48$, $P < 0.01$).

Weekly data analysis (not shown) indicated that the inulin effects on HRE became marginally significant at wk 4 ($P = 0.07$) and significant at wk 5 ($P < 0.01$). Dietary inulin concentrations had no effect on final body weight, daily body weight gain, daily feed intake, final hematocrit, or final fecal pH (Table 2).

2.4.2 Experiment 2

There was no difference in overall growth performance or final hematocrit between pigs fed BD and 4% inulin. Pigs fed 4% inulin had 14% higher (94.4 ± 4.5 vs. 107.8 ± 3.7 g/L, $P = 0.06$) blood Hb concentrations at wk 6, and 22% higher (20.4 ± 1.4 vs. 24.9 ± 0.7%, $P < 0.05$) overall HRE than that of pigs fed BD. The changes in Hb concentrations over the 6 wk period were greater ($P < 0.05$) in the pigs fed 4% inulin than those fed the BD. Although total iron concentrations of digesta from various segments were not significantly different (**Fig. 2A**), pigs fed 4% inulin had 45% higher ($P < 0.01$) soluble Fe concentrations in the three segments of colon than those of pigs fed BD (**Fig. 2B**). In contrast, digesta soluble sulfide concentration in pigs fed 4% inulin was 32% lower ($P < 0.01$) in digesta of distal colon and marginally lower (17%, $P = 0.08$) in mid colon than that of pigs fed BD (**Fig. 3**). The two groups of pigs showed no difference in the pH of digesta samples from various segments (stomach: 3.2 ± 0.2 vs. 3.3 ± 0.2; upper jejunum: 6.6 ± 0.2 vs. 6.5 ± 0.1; lower jejunum: 7.3 ± 0.1 vs. 7.2 ± 0.1; proximal colon: 6.6 ± 0.2 vs. 6.6 ± 0.2; mid colon: 6.8 ± 0.1 vs. 6.8 ± 0.03; and distal colon: 6.8 ± 0.1 vs. 6.7 ± 0.1) or fecal samples (6.0 ± 0.2 vs. 6.1 ± 0.2) at the end of the study. When phytase activity in digesta was assayed at pH 5.5, there was no

difference between the two groups of pigs in digesta from any segment except for the lower jejunum where pigs fed 4% inulin had a slightly higher activity than that of pigs fed the BD (**Table 3**). When phytase activity in digesta was assayed at the actual digesta pH of each segment, only stomach digesta showed detectable activity (BD, 52.9 ± 11.5 U/g; 4% inulin, 29.2 ± 7.5 U/g; $P = 0.10$).

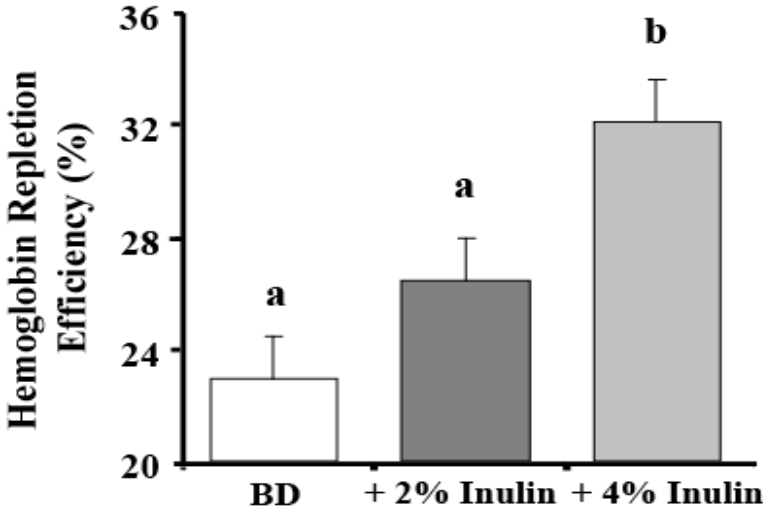

Figure 2.1 Effects of supplemental inulin on hemoglobin repletion efficiency (HRE) of pigs in Expt. 1. The calculation of HRE is described in the text(85). Values are means ± SEM, $n = 8$. Means without a common letter differ, $P \leq 0.05$.

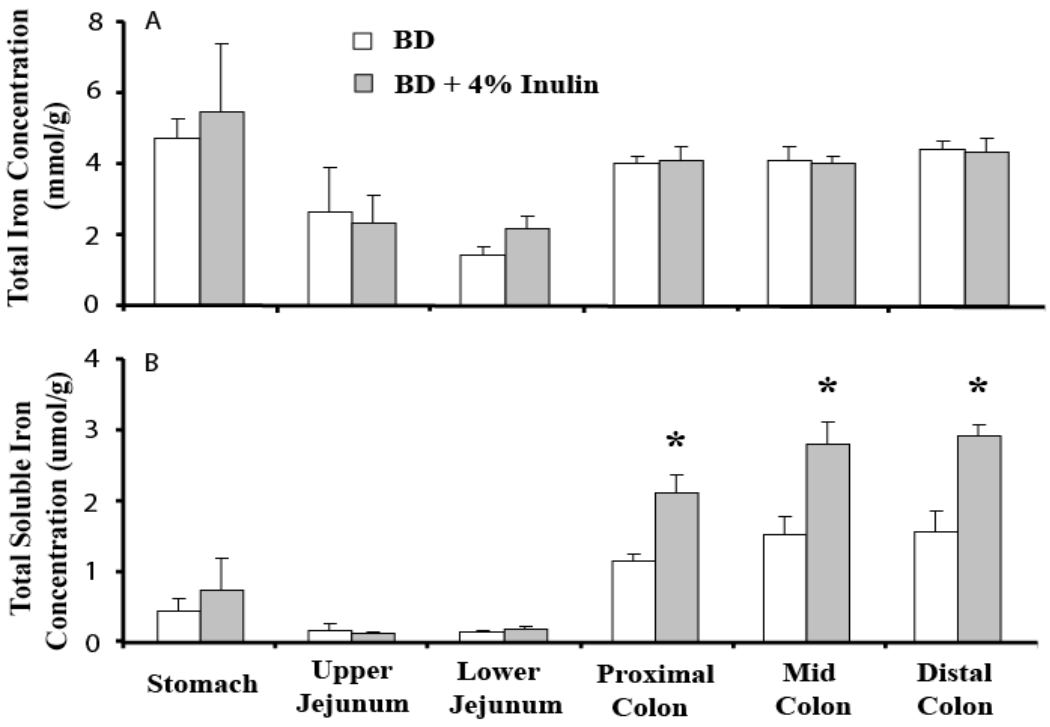

Figure 2.2 Effects of supplemental inulin on total Fe concentration (A) and soluble Fe concentration (B) of digesta of pigs in Expt. 2. Values are means ± SEM, $n = 8$. *Different from BD, $P < 0.05$.

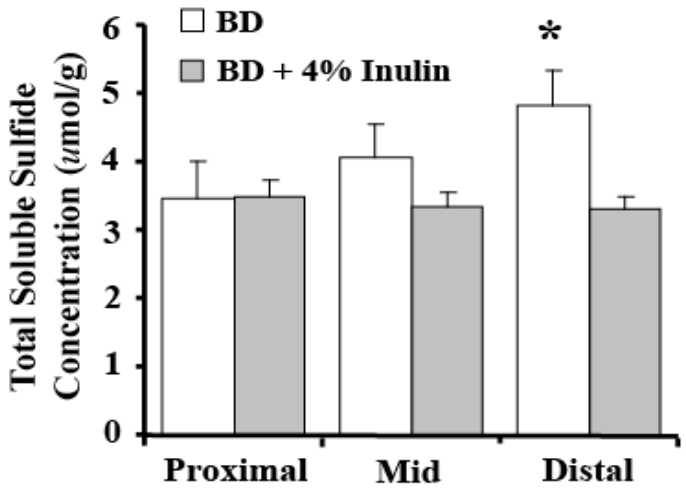

Figure 2.3 Effects of supplemental inulin on total soluble sulfide concentration of digesta of pigs in Expt. 2. Values are means ± SEM, $n = 6$. *Different from BD, $P < 0.05$.

Table 2.2 Effect of dietary supplemental inulin on growth performance, blood hemoglobin concentration, and fecal pH of pigs in Expt. 1. Values are means, $n = 8$. Means in a row with superscripts without a common letter differ, $P < 0.05$. Data were analyzed using 1-way ANOVA with (Hb, hematocrit, and body weight) or without (weight gain, feed intake, and fecal pH) time-repeated measurements.

	Time, wk	BD	+ 2% inulin	+ 4% inulin	SEM
Hemoglobin, g/L	0	77.0	73.3	73.2	1.8
	5	128.0[a]	135.2[a,b]	147.1[b]	4.0
Hematocrit	0	0.299	0.289	0.308	0.006
	5	0.374	0.370	0.376	0.009
Body weight, kg	0	9.4	9.2	9.2	0.03
	5	29.4	30.4	31.0	0.1
Weight gain, g/d	0-5	571.4	605.7	622.9	21.5
Feed intake, g/d	0-5	1091.3	1138.8	1024.8	0.3
Fecal pH	5	6.5	6.3	6.5	0.02

Table 2.3 Effect of dietary supplemental inulin on growth performance, blood hemoglobin concentration, and fecal pH of pigs in Expt. 2. Values are means, $n = 6$. Means in a row with superscripts without a common letter differ, $P < 0.05$.

	Time, wk	BD	+ 4% inulin	SEM
Hemoglobin, g/L	0	67.8	76.0	1.61
	6	93.9[a]	106.0[b]	3.24
Hematocrit	0	0.288	0.292	0.09
	6	0.318	0.328	0.14
Body weight, kg	0	8.3	8.8	0.19
	6	24.8	28.3	1.17
Weight gain, g/d	0-6	420.0	500.0	40.0
Feed intake, g/d	0-6	1130.0	1140.0	60.0

Table 2.4 Effect of dietary supplemental inulin on digesta pH of pigs in Expt. 2. There was no significant difference between treatment groups.

	BD	BD + 4% inulin	SEM
Stomach	3.26	3.26	0.20
Duodenum	6.18	6.66	0.26
Upper Jejunum	6.62	6.46	0.13
Lower Jejunum	7.27	7.20	0.09
Proximal Colon	6.64	6.57	0.17
Mid Colon	6.74	6.79	0.05
Distal Colon	6.77	6.69	0.06

Table 2.5 Effect of dietary supplemental inulin on digesta phytase activity in pigs of Expt. 2. The activities were assayed using the standard buffer (0.2 M citrate pH 5.5, and 6.0). Values are means, $n = 6$. *Different from BD, $P < 0.05$.

	BD	BD + 4% inulin	SEM
		pH 3.5	
Stomach	52.3	29.2	0.10
		pH 5.5	
Stomach	24.8	19.3	3.9
Lower Jejunum	19.1	42.5*	6.5
Proximal Colon	440.8	388.5	115.0
Mid Colon	395.6	348.0	63.1
Distal Colon	393.7	410.1	84.9
		pH 6.0	
Cecum	234.4	230.7	97.0
Lower Jejunum	12.3	20.7	9.0
Proximal Colon	376.0	432.2	72.0
Mid Colon	496.5	349.0	134.5
Distal Colon	152.8	228.5	66.2

2.5 Discussion

Supplemental inulin added to corn-soybean meal diets significantly improved bioavailability of iron from corn-soybean meal diets fed to weanling pigs. In Exp. 1, this improvement displayed a linear response to dietary inulin dose. Because pigs in both experiments were fed diets without inorganic iron addition, the improved HRE clearly indicated that supplemental 4% inulin effectively enhanced the bioavailability of iron intrinsically present in corn and soybean meal to meet the most quantitatively important function of iron in the body, hemoglobin synthesis. This finding is novel and extremely encouraging as a strategy for improving human iron nutrition through biofortification of staple food crops (66). Although ingestion of high concentrations of inulin may cause excessive flatus, borborygmi, and bloating (92), earlier studies have shown that humans may be able to tolerate inulin intakes up to 30 g/d (92). Most plants have inulin concentrations ranging from 0.1% to 3.2% (20). If our pig data are applicable to humans, it will be physiologically feasible to achieve significant improvements in iron nutrition of target human populations by enriching inulin in their staple foods via plant breeding. Differential effects of short and long chain inulin on iron bioavailability should be considered for the target enrichment. The entire plant of such new varieties would need to be thoroughly tested for possible alterations in other nutrients.

The positive effect of inulin on HRE in pigs in the present study is consistent with that of FOS in rats reported by Ohta et al. (5). However, other groups did not observe a positive effect of inulin or FOS in humans (51,68). This discrepancy does not seem to be simply explained by differences in inulin or FOS doses between these experiments. The positive effects in rats were produced by 5 to 10% inulin or FOS (76,93-95), whereas the human subjects who did not show any response were given approximately 3% (15 g of inulin/d) (8) or 8% (40 g of inulin/d) (9) inulin.

Alternatively, initial iron status of the experimental animals or subjects might be the key determinant of the treatment outcomes. Our pigs experienced moderately iron-deficient anemia and grew normally, which provided an appropriate physiological condition for inulin to show its effect on iron bioavailability. However, healthy, non-Fe-deficient subjects were used in the human studies (51,68).

Thus, supplemental inulin may exert a greater role in iron deficient animals than in iron-adequate subjects (5,68). If so, enriching inulin in staple crops may benefit the iron-deficient population without putting the iron-adequate population at risk of iron excess. As the positive effects of 4% inulin on blood Hb and HRE were not significant until Wk 5, a minimal length of time was needed for supplemental inulin to show its maximum effect. In addition, the type of inulin may affect the outcome. The inulin used in our study was a mixture of long and short chain oligofructose polymers, whereas van den Huvel et al. used short chain oligofructose consisting of glucose linked to 2 to 4 fructose units (68), and Coudray et al. used inulin of longer chain length (DP = 15) (51). Different types of inulin or FOS fare differently in the digestive tracts, and affect different types of microbial populations, leading to different digestive or metabolic impacts (76,93-95). It is also interesting to mention that soybean meal contains 4-6 % galactooligosaccharides (96). As we and others have observed an enhanced iron bioavailability by supplementing inulin or FOS into the basal diets containing up to 30% soybean meal, this again suggests the specificity of oligosaccharides in impacting select biochemical and metabolic responses.

Compared with pigs fed the BD, pigs fed 4% inulin had higher concentrations of soluble iron and lower concentrations of sulfide in digesta of the colon. To our best knowledge, the effect of inulin on digesta sulfide concentrations has not been reported, although Sakai et al. (52) observed an increased soluble iron concentration in the colon of rats fed 7.5% short-chain FOS. The increased solubility of iron in colon digesta would promote absorption of iron if mineral absorption takes place in the large intestine

(52,73,97). Although it is still a subject of debate whether significant amounts of iron can be absorbed in the colon (98), a few recent studies have shown the expression of iron absorption-related genes in the large intestines of rats and mice (99,100). Furthermore, Ohta et al. have reported that dietary inulin supplementation resulted in a positive correlation between apparent calcium absorption and the relative amounts of calbindin (calbindin-D9k) and strongly induced CaBP expression in large intestines of rats (97). Our group is actively investigating whether inulin can up-regulate an iron transporter in the colon of pigs.

As sulfide is generated from microbial fermentation (79), the reduced concentration of sulfide in the distal colon digesta may be interpreted as a modified microbial population in the colon of the inulin-fed pigs, leading to an attenuated hydrogen sulfide production (79,101). Consequently, lowering sulfide would reduce its binding to iron (102), leaving more iron soluble or available for possible absorption. Our data are in agreement with Swanson et al. (22) and Flickinger et al. (76,94) who observed a reduction in fecal hydrogen sulfide and other fecal putrefactive agents (protein fermentative catabolites) in dogs fed FOS. Reduction of such moieties, if verified, would be extremely important in human and companion animal gut health because these subjects may ingest excess protein and indoles, phenols, and S-containing compounds have large bowl disease implications (79).

Because we did not see any effect of supplemental inulin on fecal or digesta pH, and virtually no effect of supplemental inulin on phytase activity in digesta of various segments at either actual digesta pH or pH 5.5, the inulin-produced improvements in HRE of pigs was not associated with intestinal pH or digesta phytase activity. Reported changes of digesta pH by supplemental inulin or FOS have been controversial. Loh et al. showed an elevated colonic pH in pigs fed 3% inulin (103), whereas Kleessen et al. observed a decreased cecal and colonic pH in rats fed 5% short or long chain FOS (93). Meanwhile, Mikkelsen et al. found no changes of digesta pH in pigs fed 4% FOS (104).

Thus, supplemental inulin or FOS does not always affect digesta pH, and their effect on iron bioavailability is not necessarily associated with lowering intestinal pH. Although stomach digesta had detectable phytase activity at its actual pH, and lower jejunum digesta phytase activity showed an inulin effect at pH 5.5, their activities were low compared with those in colon digesta. Thus, the detected phytase activity in upper gut was due to mainly the plant phytase present in the diet, and probably did not have a major impact on iron bioavailability. Clearly, supplemental inulin did not seem to promote phytase-producing microbes in the gastrointestinal tracts of pigs.

In conclusion, our results indicate that supplemental 4% inulin improved utilization of iron intrinsically present in corn and soybean meal by young pigs for hemoglobin synthesis. This positive effect of inulin was associated with deceased concentrations of sulfide and increased concentrations of soluble iron in colon digesta, but not with digesta pH or phytase activity across different segments of the gastrointestinal tracts of pigs. The supplemental inulin concentration used in the present study is close to the tolerable threshold of humans(92), above the level that caused dramatic positive shifts in the composition of microbiota in humans(23), and achievable in staple crops by plant breeding (42). Thus, our findings are highly relevant to improving iron nutrition in anemic population through biofortifcation. The combined effectiveness of inulin with other approaches in improving iron nutrition merits future research.

CHAPTER THREE

SITE OF INULIN DISAPPEARANCE

3.1 Abstract

Two groups have reported > 90% of pre-caecal digestibility of inulin in pigs, and argued against pigs as a proper animal model for humans in this regard. Two experiments were conducted with weanling pigs to characterize the hydrolysis profile of inulin in various segments of the GI tract. In Exp. 1, 12 pigs (7.7 ± 0.2 kg of body weight) were fed a low-iron (54 mg/kg) BD or BD + 4% inulin (Synergy 1, Orafti, Tienen, Belgium) for 6 wk. All pigs were killed at the end of the trial and digesta samples were collected from the stomach, upper and lower jejunum, cecum, and proximal, mid, and distal colon. Inulin was detected in digesta from the first three segments (0.4 to 5.5%, dry basis), but not from the large intestine of pigs fed inulin. Fructose concentrations in digesta from the stomach and jejunum were greater ($P <$ 0.05) in pigs fed inulin than those fed BD. To further determine whether and how inulin was degraded in the ileum or cecum, we conducted Exp. 2 with 12 pigs (11.2 ± 1.1 kg of body weight) for 8 wk as in Exp.1. except that ileum instead of upper jejunum digesta samples were collected. Inulin was detected only in digesta from stomach, jejunum, and ileum of pigs fed inulin. Although the activity of inulin degrading enzymes was detectable in digesta from the ileum, cecum, and proximal colon of both groups, the highest activity ($P < 0.05$) was found in the cecum digesta of pigs fed inulin. Digesta from the cecum and colon, but not from the ileum, was able to degrade added inulin in *in vitro* incubations. We conclude that supplemental dietary inulin fed to pigs was degraded mainly in the cecum. Similar to humans, pigs had very limited capacity for hydrolysis of ingested inulin in the upper GI tract from stomach to ileum.

3.2 Introduction

Due to their reported beneficial effects to host, inulin and oligosaccharides have attracted tremendous interest. Inulin and oligosaccharides are types of β(2-1) fructans, consisting of varying numbers of fructose residues bonded by fructosyl-fructose linkages (16). Progress has been made in understanding their general effects on the colonic microflora (32,36,76,105), subsequent metabolites (36,76,94,106), and mineral bioavailability (5,6,44,70). Accordingly, our studies have shown a positive effect of supplemental inulin on the bioavailability of intrinsic Fe in a corn-soybean meal in weanling pigs (4).

A validated model(s) that bypasses the burdens and limitations of human trials is essential to facilitate further exploration on the likely potentials of inulin, oligosaccharides, and other fructans. Pigs and humans have been known to have similar digestive anatomy and physiology (60). However, the currently available data on inulin degradation in the gastrointestinal (G.I.) tract shows surprising differences between the two models. While inulin and oligosaccharides are digested (90-100%) before reaching the colon in pigs (30,31), virtually no degradation (26-29,61,107-109) or absorption (62,63) occurs before the colon in humans. Previous pig studies may have fallen short of precisely identifying the location of degradation due to technical limitations in sample collection, and in measurements of inulin levels. We addressed the need to apply a more direct and sensitive method to evaluate inulin degradation in the gastrointestinal tract in these studies.

High levels of selective types of colonic bacteria, namely bifidobacteria (36,78), and to a lesser extent, lactobacillus (76), are the presumed hallmarks of active sites of inulin degradation. Accordingly, *in vitro* studies have shown a direct correlation between number of Bifidobacteria and active levels of inulin degrading enzymes (110-112). It is possible that ingested inulin stimulates the proliferation of Bifidobacteria,

which may result in the increased production and activity of enzymes responsible for degrading inulin and oligosaccharides within the G.I. tract.

Data elucidating the effects of inulin and oligosaccharides on dry matter/water content (29,105,113) and the rate of passage of digesta (26,94), key determinants of nutrient bioavailability, have been inconsistent across several studies. Deviations in results may be due to the different types and amounts of fructans used. Thus a reevaluation of these fundamental properties is necessary in our pig models.

The prime objectives of this study were to identify the fate of ingested inulin and soybean oligosaccharides in the digestive tracts, and to measure G.I. tract inulin degrading enzyme activity, digesta dry matter content and rate of passage of digesta.

3.3 Materials and Methods

This chapter discusses the fate of ingested inulin in various segments of G.I. tract of pigs. Data from the present study Expt. 1 is described as Expt. 2 elsewhere (4) including the diets, growth performance, feed and blood analysis and digesta sampling. The experimental procedures were approved by the University Institutional Animal Care and Use Committee.

3.3.1 Basal diet and Inulin

The diets and inulin used in Expt. 2 were same as Expt. 1, except that it contained adequate Fe (110 mg/kg: Fe supplemented at 100 mg/kg diet as means of $FeSO_4 \cdot 7H_2O$ (Sigma. Cat. # F7002)). Synergy 1 (Orafti, Tienen, Belgium) was used as the source of inulin replacing corn starch in the basal diet (114). This product was a mixture of oligofructose (mean DP = 4) and long-chain inulin HP (mean DP = 25).

3.3.2 Experimental Animals and Protocols

Two experiments were conducted with a total of 24 weanling Yorkshire × Hampshire × Landrace crossbred pigs from the Cornell University Swine Farm. All experimental pigs were selected from litters that were injected with only a half of the normal iron dose (50 mg of iron as Fe-dextran) at birth, and were allocated to treatment groups based on body weight, litter and gender. Pigs were penned individually in Expt. 1 and group-penned in Expt. 2, in an environmentally controlled barn (22 ~ 25^{o}C; a light:dark cycle of 12:12 h). All pigs were given free access to feed and water, and checked daily. In each experiment, 12 weanling pigs (with body weights of 7.70 ± 0.2 kg) were allotted into two groups (n = 6), and were fed the BD or BD + 4% inulin for 6 and 8 wk, respectively. Prior to the beginning of both experiments, all pigs were fed the BD for 2 wk to adjust their body iron stores.

3.3.3 Growth Performance and Sample Collection

In both experiments, feed intake (individual pigs in Expt. 1 and group in Expt. 2) were recorded daily and body weight of individual pigs was measured weekly. Blood samples of all pigs (fasted overnight for 8 h) were collected weekly from the anterior vena cava using 5-mL heparin syringes to assay for blood Hb and hematocrit. Pigs were fasted, and slaughtered, and digesta samples were collected from stomach, upper and lower jejunum, cecum, proximal, mid and distal colon in Expt. 1 (4). In Expt. 2, ileum samples, a 12 cm segment directly anterior to the ileal-cecal junction were collected instead of upper jejunum (Expt. 1) to further determine if and how inulin was degraded mainly in the ileum or cecum. Digesta samples were first freeze dried (20 SRC-X, Virtis Co. Inc., Gardiner, NY) and were ground with a coffee grinder in a cold room

(4°C). Digesta dry matter contents were determined by calculating the weight difference before and after freeze drying.

3.3.4 Diet and Digesta Sugar Analysis

The concentrations of inulin, glucose, fructose, lactose, sucrose, raffinose, stachyose, and verbascose of diets and of digesta samples were determined using the method described by Quemer et al. (84). Digesta samples were auto-claved in distilled water to destroy sugar degrading microbes before proceeding to the standard method. It has been confirmed that auto-clave does not degrade significant levels of inulin and other sugars (data not shown). For inulin, the supernatant fractions of before and after complete enzymatic hydrolysis were analyzed by high performance anion-exchange chromatography (Dionex, Sunnyvale, CA). The system consisted of a gradient pump and programmable pulsed electrochemical detector (ED50 Electrochemical Detector, Dionex). Separations were performed using a Carbopac PA100 column (4x250mm) and columns were preceded by a Dionex GM-4 gradient mixer. The isocratic point of the chromatographic mobile phase consisted of 150 mM NaOH for 0 ~ 5 min and the gradient was made to 300 mM NaOH for 5 ~ 12 min.

3.3.5 In vitro Incubation of Inulin by Digesta

Digesta samples from the ileum, cecum and proximal colon from Expt. 2 were used to determine the rate of inulin degradation in each segment. 0.2 g of freeze-dried digesta samples were suspended in 2.0-ml of ice cooled distilled water using disposable glass tubes (16 x 100, Fisher Scientific). Control samples were autoclaved for the same reason as stated above. Inulin solution was then added to make 4% final inulin (w/v) concentration to all samples. Samples were then incubated aerobically in a water bath

at 37^{o}C for 0, 1, and 4 h: stirred every 30 min. At each termination time, samples were removed from the water bath and placed in – 80^{o}C freezer. All samples were freeze-dried for 48 h and stored in a - 20 ^{o}C freezer until analysis. The inulin concentrations of these samples were measured using the same method described above for the digesta.

3.3.6 Inulin Degrading Enzyme Extraction and Purification

Weigh a 1g digesta sample into a 15-ml falcon tube (Becton Dickinson, Oxnard, CA). Add 10 ml of 55 *mM* 2 *N*-morpholino-ethanesulphonic acid buffer (pH 5.5) into the tubes. Samples were sonicated and enzymes were extracted by constant stirring (magnetic stir bar) at 4^{o}C for 30 min. Transfer the mixture into a 50-mL Falcon tube and centrifuge it at 4^{o}C and 15,000 x g for 20 min with the GA-20 rotor (GS-6KR Centrifuge, Beckman Instruments Inc.). Transfer the supernatant fraction (use a 25-mL disposable pipette) to a conical tube. Spin column method was used to remove free sugars: pipette 0.5 mL of filtrate into the sample reservoir of the spin column and centrifuge the assembly at 14,000 x g at 4 ^{o}C up to final volume of approximate 200 uL (approximately 90 min, depends of tissue samples). Using a new tube weigh the retentate and add 55 *mM* 2 *N*-morpholino-ethanesulphonic acid buffer (pH 5.5) to make a final volume of exactly 0.2 mL. Mix the resultant solution thoroughly for the subsequent hydrolysis activity assay.

3.3.7 Enzyme Hydrolysis Assay

Digesta inulinase activities were measured using a protocol described by Buert et al. (Cite). The amount of NADPH formed was measured at 340 nm using KC-4 version 2.6 microplate scanning spectrophotometer (BIO-TEK® Instruments, Inc. Winooski, VT). The activity is expressed as mg sugar liberated/h.

3.3.8 Determining the Rate of Passage

Chromic oxide (Chromium Oxide Powder (Cr_2O_3), J.T. Baker Inc., Phillipsburg, NJ) was used as a marker to determine the rate of passage (115). Pigs were fasted for 8h prior to the feeding of 0.2% Cr containing diets. Rate of passage is described as measuring the time feeds were offered to when the chromium was first observed in feces.

3.3.9 Statistical Analysis

Data were analyzed as a randomized block design using the Proc General Linear Models procedure of SAS (version 6.12, SAS Inst., Inc., Cary, NC). Effects of dietary inulin on various measures were analyzed using one-way ANOVA with or without time-repeated measurements. Each individually-penned pig and each group penned pigs were used as the experimental unit in Expt 1 and 2, respectively. The *Boneferroni t*-test was used to compare treatment means, and the significance level was set at $P \leq 0.05$ (91). Sucrose, stachyose, and raffinose amounts were log transformed prior to the *Boneferroni t*-test. Values in the text are means ± SEM.

3.4 Results

3.4.1 Experiment 1

There was no difference in overall growth performance between pigs fed BD and 4% inulin. Compared to pigs fed BD, pigs fed 4% inulin had 84%, 99%, and 97% higher ($P < 0.05$) inulin contents in the stomach (0.06 ± 0.1 vs. 0.38 ± 0.2%), upper jejunum (0.02 ± 0.01 vs. 2.80 ± 0.8%) and lower jejunum (0.16 0.1 ± vs. 5.50 ± 1.5%),

respectively (**Fig. 1**). Inulin was, however, not detected in the rest of the gastrointestinal tracts: cecum, proximal colon, mid colon, and distal colon. Digeta sugars such as, glucose, fructose, sucrose, raffinose, stachyose and verbascose were also not detected in the cecum, proximal colon, mid colon, and distal colon, except for low amounts of fructose (0.2 ± 0.08 μmol/g) and sucrose (0.3 ± 0.03 μmol/g) in the cecum of pigs from both groups. Compared to BD fed pigs, pigs fed 4% inulin had 60%, 61%, and 97% higher ($P < 0.05$) fructose concentrations in the stomach (10.1 ± 3.1 vs. 25.3 ± 2.7 μmol/g), upper jejunum (22.0 ± 5.0 vs. 56.4 ± 17.8 μmol/g) and lower jejunum (0.4 ± 0.1 vs. 13.1 ± 1.8 μmol/g), and had 74% and 99% higher ($P < 0.05$) concentrations of raffinose (1.0 ± 0.7 vs. 4.0 ± 1.7 μmol/g) and stachyose (0.1 ± 0.01 vs. 6.2 ± 2.2 μmol/g) in the lower jejunum, respectively. All other sugars and segments that are not listed above shared very similar concentrations: glucose (stomach: 266.2 ± 106.4 vs. 199.1 ± 121.3 μmol/g; upper jejunum: 48.6 ± 11.2 vs. 34.8 ± 20.3 μmol/g; lower jejunum: 3.7 ± 2.6 vs. 6.1 ± 1.7 μmol/g), sucrose (stomach: 14.6 ± 2.4 vs. 12.0 ± 2.3 μmol/g; upper jejunum: 7.5 ± 2.4 vs. 10.3 ± 2.3 μmol/g; lower jejunum: 11.4 ± 10.6 vs. 10.2 ± 5.1 μmol/g), raffinose (stomach: 9.6 ± 3.3 vs. 4.7 ± 3.6 μmol/g; upper jejunum: 9.9 ± 2.5 vs. 8.7 ± 2.0 μmol/g), stachyose (stomach: 4.2 ± 0.5 vs. 2.6 ± 0.9 μmol/g; upper jejunum: 16.8 ± 5.4 vs. 19.7 ± 5.2 μmol/g) and verbascose (stomach: 0.01 ± 0.01 vs. 0.03 ± 0.01 μmol/g; upper jejunum: 0.3 ± 0.01 vs. 0.4 ± 0.2 μmol/g; lower jejunum: 0.4 ± 0.01 vs. 0.6 ± 0.3 μmol/g) between the two groups. Lactose was not detected in the diets and any of the digesta samples. The rate of passage of digesta (918 min ± 58min) and digesta dry matter contents of various segments (stomach: 17.3 ± 1.8 vs. 12.9 ± 4.4%; upper jejunum: 13.3 ± 3.1 vs. 12.4 ± 2.4%; lower jejunum: 15.9 ± 1.3% vs. 14.2 ± 0.8%; proximal colon: 20.4 ± 0.8 vs. 18.1 ± 0.6%; mid colon: 24.3 ± 0.5 vs. 24.2 ± 0.8%; distal colon: 25.4 ± 0.9% vs. 23.2 ± 0.9%) did not differ between the two groups.

3.4.2 Experiment 2

There was no difference in overall growth performance between pigs fed BD and 4% inulin. Compared to pigs fed the BD, pigs fed 4% inulin had higher ($P < 0.05$) concentrations of inulin in the ileum (0.0 ± 0.0 vs. 2.18 ± 0.6%), a segment that was not collected in Expt. 1 (**Fig. 2**). Other segments shared similar inulin profile to that of Expt. 1, where 4% inulin fed pigs had higher ($P < 0.05$) concentrations of inulin in the stomach (0.20 ± 0.1 vs. 0.94 ± 0.2%) and lower jejunum (0.26 ± 0.1 vs. 2.65 ± 0.6%) compared to pigs fed the BD. Again inulin was not detected in the cecum, proximal colon, mid colon, and distal colon of the two groups. Compared to pigs fed the BD, pigs fed 4% inulin had 80%, 85%, and 88% higher ($P < 0.05$) concentrations of digesta fructose in the stomach (2.0 ± 0.5 vs. 9.9 ± 4.1 μmol/g), lower jejunum (1.0 ± 0.4 vs. 7.0 ± 1.8 μmol/g), and cecum (0.1 ± 0.0 vs. 0.9 ± 0.2 μmol/g), respectively and 89% higher ($P < 0.05$) concentrations of sucrose (4.9 ± 1.0 vs. 43.1 ± 13.0 μmol/g) in the ileum (**Fig. 3**). In contrast, 4% inulin fed pigs had 61%, and 88% lower glucose concentrations in the stomach and cecum than pigs fed the BD. Although, ileum had low levels of sucrose (3.8 ± 1.1 vs. 2.3 ± 1.3 μmol/g) and raffinose (0.1 ± 0.1 vs. 0.4 ± 0.5 μmol/g), other sugars were not detected in both groups of pigs. Virtually no sugars were again detected in three segments of the colon of pigs in both groups. When inulin was incubated with digesta from the ileum, both the BD and 4% inulin fed pigs shared similar trend and were not different from hour 0 (**Fig. 4**). In contrast, when inulin was incubated with digesta from the cecum , 4% inulin fed pigs had faster rate of inulin degradation: where 4% inulin, but not the BD fed pigs showed reduction ($P < 0.05$) of inulin at hour 1, than pigs fed the BD. Moreover, the relative rate of fermentation was similar for both BD and 4% inulin fed pigs when colon samples were incubated with inulin. The levels of inulin degrading enzymes were similar between the two groups in the ileum and proximal colon. Compared to pigs fed BD, 4% inulin fed pigs had 4-fold

higher ($P < 0.05$) activity in the cecum (**Fig. 5**). The two groups of pigs did not differ in dry matter digesta content from any segments (stomach: 22.2 ± 0.5 vs. 21.6 ± 0.2%; lower jejunum: 12.1 ± 1.2 vs. 12.9 ± 0.5%; ileum 11.7 ± 1.2 vs. 11.5 ± 0.5%; cecum: 11.2 ± 0.7 vs. 12.1 ± 0.3%; mid colon: 21.9 ± 0.4 vs. 20.2 ± 0.2%; distal colon: 23.0 ± 0.9 vs. 21.8 ± 0.4%) except for proximal colon, where pigs fed 4% inulin had slightly higher ($P < 0.05$) dry matter content (12.4 ± 0.7 vs. 15.6 ± 0.3%) than pigs fed the BD. The rate of passage (798 ± 26 vs. 767 ± 30 min) did not differ between the two groups.

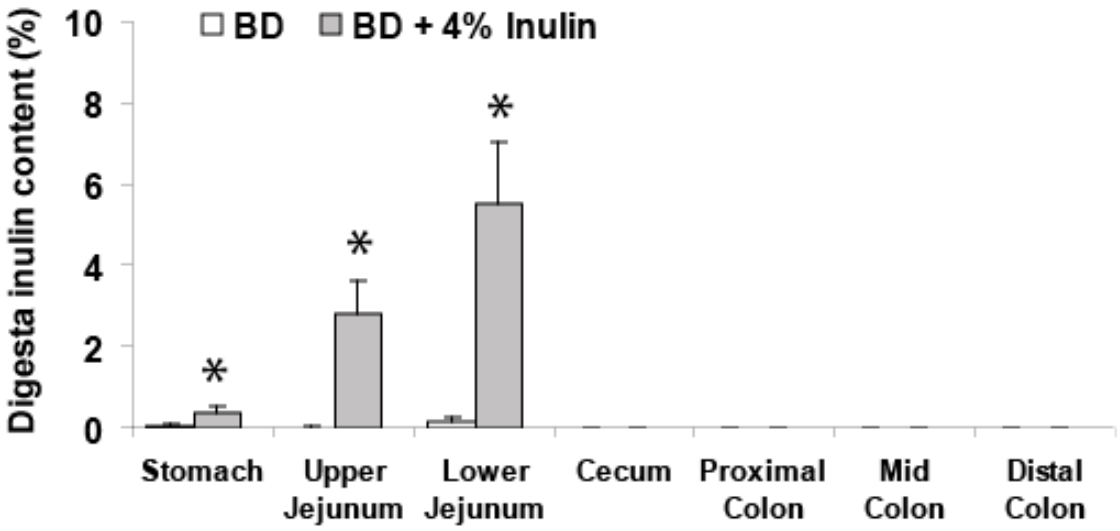

Figure 3.1 Digesta inulin contents of pigs in Expt. 1. Values are means ± SEM, n = 6. Data were analyzed using 1-way ANOVA. *Different from BD, $P < 0.05$.

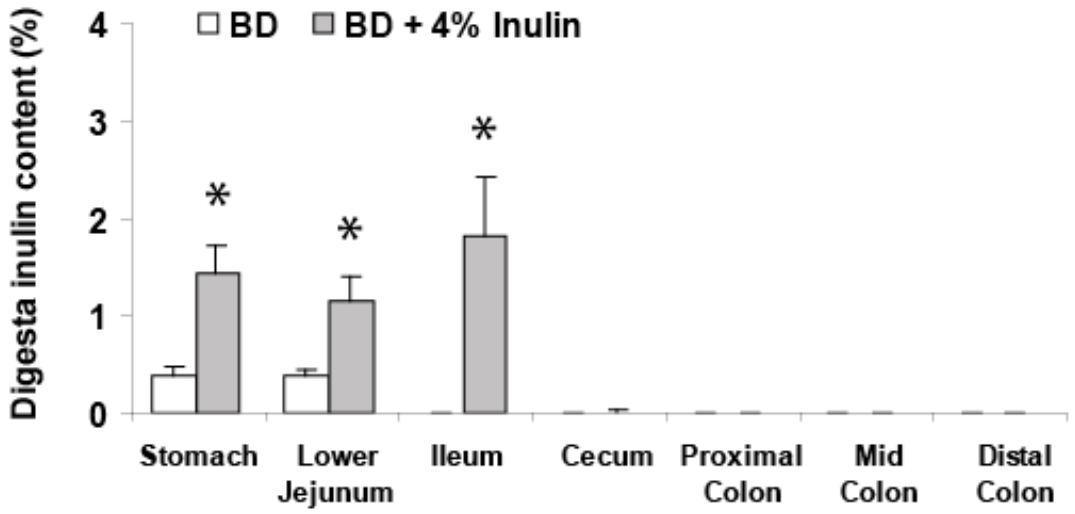

Figure 3.2 Digesta inulin contents of pigs in Expt. 2. Values are means ± SEM, n = 6. Data were analyzed using 1-way ANOVA. *Different from BD, $P < 0.05$.

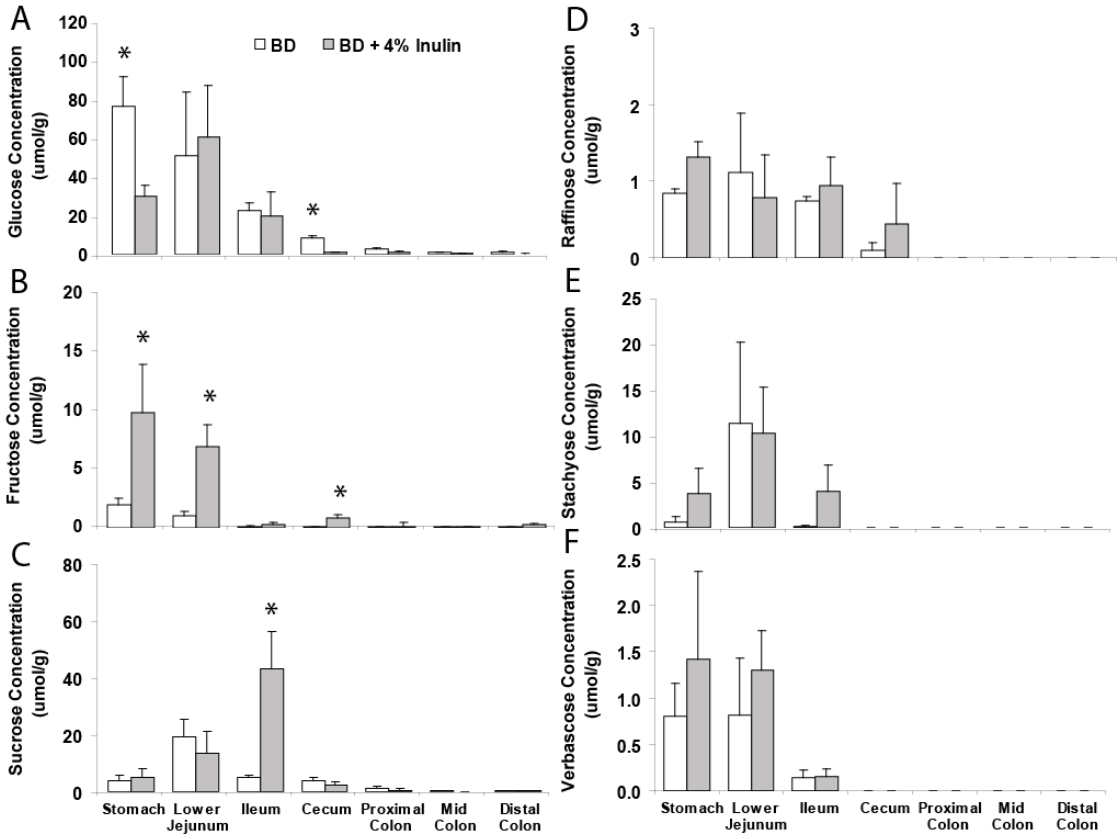

Figure 3.3 Digesta concentrations of glucose (A), fructose (B), sucrose (C), raffinose (D), stachyose (E), and verbascose (F) of pigs in Expt. 2. Data were analyzed using 1-way ANOVA. Values are means ± SEM, $n = 3 \sim 6$. *Different from BD, $P < 0.05$.

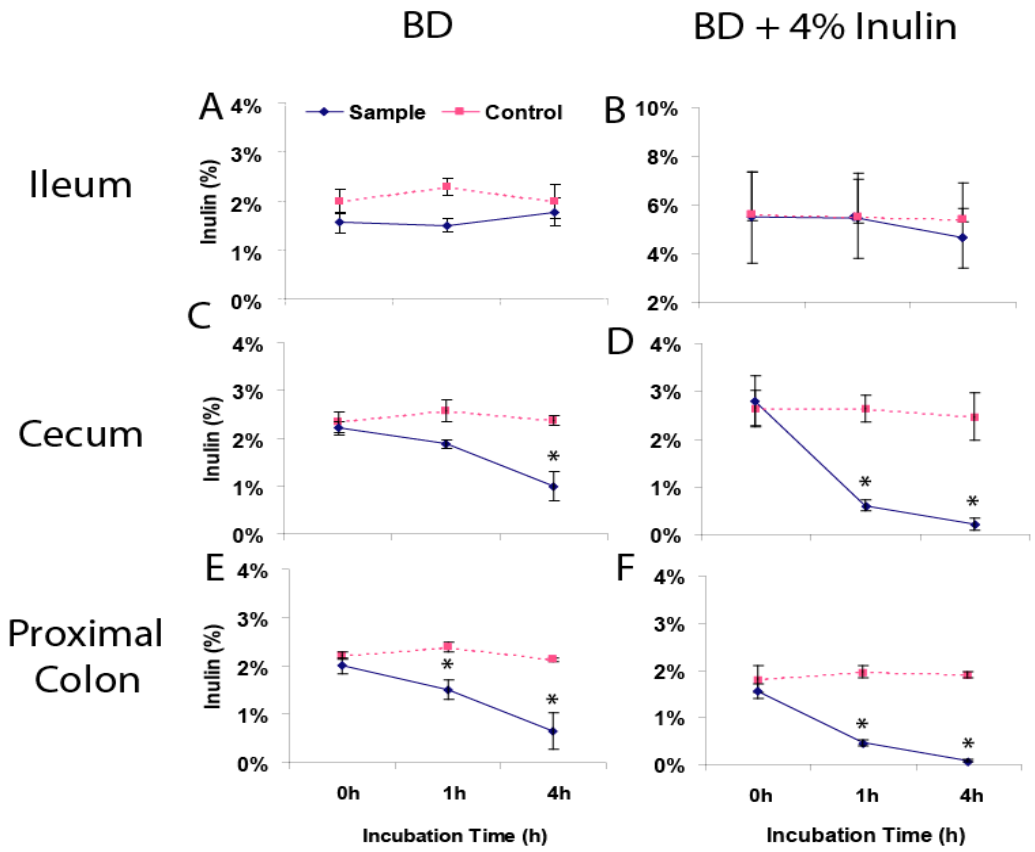

Figure 3.4 In vitro incubation of inulin by ileum (A) and (B), cecum (C) and (D) and proximal colon (E) and (F) digesta of pigs fed BD and BD + 4 % inulin diet, respectively, of Expt. 2. 0.2 g of freeze-dried digesta samples were suspended in 2-ml of ice cooled distilled water. Control samples (---) were autoclaved then. Inulin solution was then added to make 4% inulin (w/v) mixed to all samples. Samples were then incubated in water bath at 37^{o}C for 0, 1, 4 h: stirred at every 30 min. At each termination time, samples were removed from water bath and placed in –80 ^{o}C freezer. Samples were kept in –80 ^{o}C freezer for 1 h and they were freeze dried and inulin contents were measured following the same methods described above. Data were analyzed using 1-way ANOVA. Values are means ± SEM, n = 3. *Different from 0h, P < 0.05.

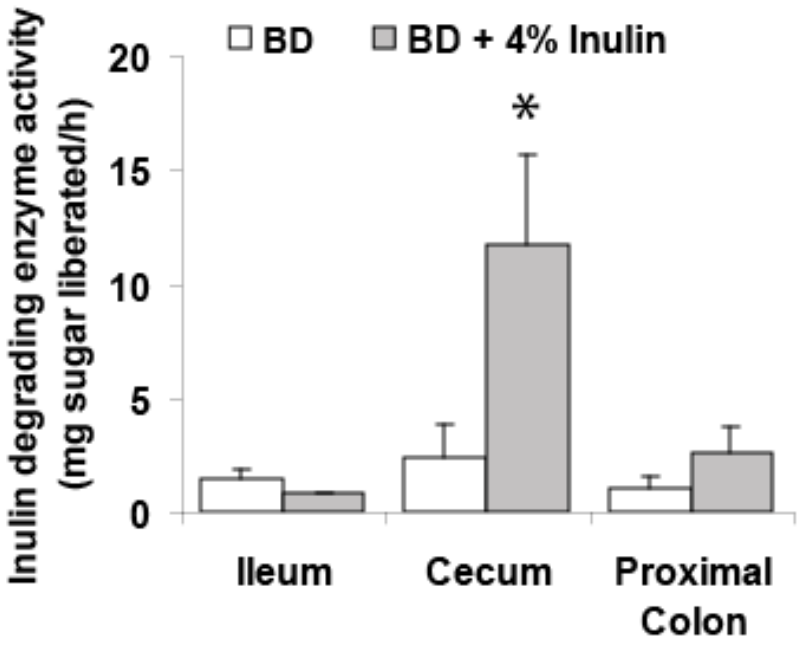

Figure 3.5 Effect of supplemental inulin on digesta inulin degrading enzyme activity of pigs in Expt. 2. Values are means means ± SEM, $n = 6$. Data were analyzed using 1-way ANOVA. *Different from BD and BD + 4% inulin of ileum and proximal colon, $P < 0.05$.

3.5. Discussion

To best of our knowledge, this is the first study to report the complete profile of ingested inulin in the G.I. tract of pigs. The sudden disappearance of ingested inulin from the ileum to the cecum, and its detection in the upper G.I. tract, but not in the cecum or thereafter, our data highly suggests that the cecum is the major degradation site of inulin in the G.I. tract of pigs.

Our data is in agreement with human studies. Bach Kundsen et al, demonstrated 87% recovery of the ingested short chain inulin (68% DP < 10) in the ileal effluent of ileostomy subjects (26). In another study using ileostomy subjects showed 88 and 89% recovery of both inulin (DP = 10) and oligosaccharides (DP = 4), respectively (29). Our findings are novel and extremely encouraging as pigs may be used as human models to study the likely potentials of inulin. In the current study, however, we were unable to quantify the recovery of ingested inulin in the small intestine. Thus, future studies may be conducted to elucidate this issue by placing a cannula to the ileum, the segment in which inulin was detected in the present study.

Although other studies using pigs indicate that inulin is digested (90-100%) before reaching the colon (30,31), our current observations certainly do not agree. This discrepancy may be explained by differences in the methodologies used. Houdijk et al, showed a complete degradation of oligosaccharides (DP = 4) in digesta, as collected through a T-cannula placed to the cecum (31). Branner et al, showed a complete disappearance of inulin (DP = 23) in digesta collected from the anus of pigs that underwent a surgical procedure which connected the terminal ileum to the proximal section of the rectum, while 20 – 25 cm of rectum remained intact (30). In our study, however, we used a slaughtering technique to collect digesta directly from the segments of interest.

Technical limitations also existed during the quantification of inulin. In our study, a high performance anion-exchange chromatograph was used, where simple sugars were evaluated through a column. The column essentially separates, identifies and quantifies the sugars by using their physical-chemical characteristics. The Houdijk et al study, on the other hand, simply subtracted the basic nutrient components (ash, crude protein, ether extract, crude fiber and protein) from the dry matter in order to derive inulin content (31). Moreover, Mikkelsen et al reported that, to an extent, fermentation takes place in the distal small intestine (apparent ileal digestibility of inulin = 57%), data obtained by incubating inulin with ileal digesta, using an enzyme reduction-coupled method to detect simple sugars (75). This technique has been shown to yield less consistent and precise results than the HPLC method used in our study (116).

I*n vitro* incubation further confirms the cecum as a major site of inulin degradation; the ileum digesta showed low, or virtually no capacity, to degrade inulin, yet the cecum and the proximal colon digesta demonstrated strong ability to degrade inulin (see Figure 4). Our *in vitro* incubation was performed under aerobic conditions, where the strict anarobes responsible for degrading inulin may not be functional.

Activity of inulin degrading enzymes was highest in the cecum of the 4% inulin fed pigs. Considering the reported information: inulin stimulates the proliferation of Bifidobacteria (36,78), a direct correlation between the number of Bifidobacteria and activity levels of inulin degrading enzymes (110-112,117), and our current data; higher inulin degrading enzyme activity in the cecum of inulin fed pigs, we have shown that the cecum may be the major site for the degradation of inulin.

Our data on soybean oligosaccharides in the G.I. tract agree with Smiricky et al. (96), who observed recovery of raffinose and stachyose in the ileum of pigs by using an ileal cannula. Interestingly, Smiricky et al. supplemented raffinose and stachyose in the diet to observe colonic fermentation (105). Therefore, it would be extremely interesting

to evaluate whether these polysaccharides show selective stimulation of growth and/or activity of beneficial bacteria (2). Such findings may clarify whether soybean oligosaccharides fulfill the criteria to be classified as probiotic.

In the ileum, stachyose and raffinose in Expt. 1 and sucrose in Expt. 2 had higher concentrations in pigs fed inulin. Because several factors can cause such a response, the exact mechanism to justify this effect is not clear from the current study only. Factors responsible for these results may include an increased viscosity of digesta, which can interfere with digestion of nutrients by decreasing their interaction with digestive enzymes (118), reduced activity of sucrase, and/or elevated or reduced hydrolysis of stachyose (2-galactose, glucose, fructose) and/or raffinose (galactose, fructose, glucose) producing sucrose (glucose, fructose) (119). Therefore, future research needs to address and reveal the cause(s) of elevated sugars in the ileum of inulin fed pigs.

Since the diets used in this study only differed by the supplementation of inulin (fructose polymers) and corn starch to replace inulin (glucose polymers), the higher concentrations of fructose in the small intestine of inulin fed pigs indicates a certain degree of inulin degradation. Because pH of the small intestine is too high for acid hydrolysis to take place (107), and that animals lack gastrointestinal and pancreatic enzymes to hydrolyze inulin (120), release of fructose is preferably consequence of small intestinal microbial fermentation. Thus, it would be interesting to investigate the effect of inulin on the profiles of such microbes. High concentrations of free fructose in the small intestine have another implication, however. It has been reported that fructose forms a stable ferric fructose chelate to enhance Fe bioavailability (121,122). Presumably, fructose liberated from inulin in the upper G.I. tract may form a stable complex with Fe (III) molecules, which may contribute to improving Fe bioavailability.

Supplementation of 4% inulin yielded no observable changes in the rate of passage and in dry matter content of digesta. These factors, therefore, did not play a major role in improving Fe bioavailability, as observed previously (4).

In conclusion, our results indicated that ingested inulin at 4% was mainly degraded in the cecum of pigs. This was in agreement with our *in vitro* incubation data and the activity of inulin degrading enzymes. Because free fructose in the small intestine may form a stable/soluble complex with iron, this mechanism may be an important factor in the improved Fe bioavailability previously shown by our group. In the current study, we were unable to distinguish between the short (oligosaccharides) and long chain inulin (DP = 25) and how they fare differently in the digestive tracts of pigs, particularly in the upper digestive tract. Our group is actively investigating this issue. Future studies need to be conducted to reveal how supplemental inulin influences small intestinal microbes, and to investigate the factors affecting Fe metabolism.

CHAPTER FOUR

COMPARISON OF THREE TYPES OF INULIN

4.1 Abstract

We have previously showed an improvement in bioavailability of dietary Fe for hemoglobin repletion by supplementation with Synergy 1 inulin in weanling pigs. This experiment was conducted to compare the efficacy of three types of inulin on iron bioavailability in weanling pigs. Thirty-two weanling pigs (BW = 9.15 ± 0.41 kg) were individually housed and fed a corn-soybean meal based diet (no inorganic Fe: 101 mg Fe/kg) with the diet supplemented with 4%: Synergy 1, HP, or P95 or without for five weeks (n = 8). Compared with those fed the basal diet (BD), pigs fed the Synergy1 (10%), P95 (9%) and HP (10%) had higher ($P < 0.05$) hemoglobin concentrations at the end of the trial. Synergy 1 fed pigs had higher ($P < 0.05$) hemoglobin repletion efficiency (HRE) than the BD fed pigs and the P95 fed pigs had the lowest ($P < 0.05$) HRE. Our finding suggested that different chain-length inulin may improve dietary iron utilization for hemoglobin repletion in pigs through different mechanisms.

4.2 Introduction

Inulin is a unique D-fructofuranose polymer linked by a β(2-1) bond, and is found in tissues of many plant species (17). Out of several commercialized inulin-type fructans, chicory inulin (degree of polymerization (DP) ranges from 2 to 60, DP_{av} = 12), oligofructose (enzymatic hydrolysis of inulin, DP = 2 - 7 and DP_{av} = 4), and short-chain fructooligosaccharides (Sc-FOS: synthetically produced by the addition of fructose units onto the fructose end of sucrose to produce 1-kestose (GF_2), nystose (GF_3) and 1f-β-fructofuranosylnystose (GF_4)) (123), have been examined for their potential health beneficial effects. Long-chain inulin (separation of oligofructose from chicory inulin, DP = 10 – 60, DP_{av} = 25; inulin HP) and Synergy 1® (50:50 mixture of oligofructose and long-chain inulin) appeared recently into the market and our knowledge on them are scarce.

Supplementation of inulin-type fructans has been shown to enhance bioavailability of dietary minerals, especially Ca and Mg, in humans (6,51), and animals (5,6,44-46,70,95,124). Using young anemic pigs as a model of humans, we have demonstrated a positive effect of supplemental inulin (Synergy 1®) on dietary iron bioavailability (4). However, little is known on the mechanisms for these benefits of supplemental inulin (4,5) and the differences in efficacy on dietary iron bioavailability between various inulin-type fructans (33).

There have been only a few studies comparing the efficacy of inulin-type fructans of different chain length on iron bioavailability (44,125). Consequently, several major issues remain to be clarified. First, the effect of long chain inulin (DP_{av} = 25) on dietary iron utilization have not been studied as Delzenne et al and Sakai et al, used

either oligofructose, Sc-FOS and inulin (Raftiline®, DP_{av} = 10), and/or inulin (Raftiline®, DP_{av} = 10). Second, only rats were used in both experiments and that rats used in Sakai's experiment underwent surgical removal of stomach and/or cecum (125), which do not represent a good model for a normal human subject. Lastly, none of the studies had clear explanations as to how these various types of inulin-related fructans affected the bioavailability of iron.

Therefore, our objectives were to determine: 1) how different types of inulin-type fructans affect the bioavailability of iron, and 2) affected soluble sulfide and Fe concentrations, dry-matter contents, feed transit time, and pH of digesta from different segments of the gastrointestinal tracts of young pigs.

4.3 Materials and Methods

4.3.1 Diets and Inulin

The basal diet consisted of corn and soybean meal (**Table 1**), and contained adequate concentrations of all nutrients (82) except for iron (no inorganic iron was added). All inulin products were provided by Orafti, Tienen, Belgium. Raftilose® P95, oligofructose α-D-fructopyranosyl-(ß-D-fructofuranosyl)$_{n-1}$- ß-D-fructofuranosides (*n* = 2 to 7, average of 4); Raftiline® HP, α-D-glucopyranosyl-(ß-D-fructofuranosyl)$_{n-1}$- ß-D-fructofuranosides (*n* = 10 to 60, average of 25) and Synergy® 1, 50:50 mixture of P95 and HP. Raftilose is produced by partial enzymatic hydrolysis of chicory inulin. Raftiline is chicory inulin devoid of fructans with the lower degree of polymerization.

4.3.2 Experimental Animals, Protocol and Sample Collection

Experiment was conducted with a total of 32 weanling Yorkshire × Hampshire × Landrace crossbred pigs from the Cornell University Swine Farm. The experiment was approved by the University Institutional Animal Care and Use Committee. All experimental pigs were selected at 6-wk after birth from litters that were injected with only a half of the normal iron dose (50 mg of iron as Fe-dextran) at birth, and were allocated to treatment groups based on body weight, litter, gender, and hemoglobin concentrations in a temperature-controlled barn (22 ~ 25^{o}C) with a light:dark cycle of 12:12 h, given free access to feed and water, and checked daily. Pigs (body weight = 9.15 ± 0.41 kg) were allotted into four groups (n = 8), and were fed the BD, BD + 4% Synergy 1, BD + 4% HP or BD + 4% P95 for 5 wk. Prior to the beginning of the experiment, pigs were fed the BD for 2 wk to adjust their body iron stores. Digesta samples were collected from stomach, lower jejunum, ileum, cecum, proximal, mid and distal colon. Procedures for blood analyses, digesta sample collection and analyses, and feed analyses are described elsewhere (4).

4.3.3 Statistical Analyses

Data were analyzed as a randomized block design using the Proc General Linear Models procedure of SAS (version 6.12, SAS Inst., Inc., Cary, NC). Effects of dietary inulin on various measures were analyzed using one-way ANOVA with or without time-repeated measurements. Each individually-penned pig was used as the experimental unit. The *Boneferroni t*-test was used to compare treatment means, and the significance level was set at $P \leq 0.05$ (91). Values in the text are means ± SEM.

Table 4.1 Composition of the experimental diets

Ingredient	BD	Synergy 1	HP	P95
		g/kg		
Corn	591.4	591.4	591.4	591.4
Soybean meal, 48% CP	306.5	306.5	306.5	306.5
Corn Oil	10.0	10.0	10.0	10.0
Corn Starch	40.0	0.0	0.0	0.0
Sodium Phosphate	11.1	11.1	11.1	11.1
Calcium Carbonate	14.5	14.5	14.5	14.5
Plasma Spray	10.0	10.0	10.0	10.0
Sodium Chloride	2.5	2.5	2.5	2.5
Vitamin/mineral Premix[1]	10.0	10.0	10.0	10.0
L-Lysine	2.5	2.5	2.5	2.5
DL-Methionine	1.0	1.0	1.0	1.0
L-Threonine	0.5	0.5	0.5	0.5
Tylan 10	5.0	5.0	5.0	5.0
Inulin	0.0	40.0	40.0	40.0
Total	1000	1000	1000	1000
Nutritional values				
ME[2], *MJ/kg*	13.8	13.2	13.2	13.2
Crude protein[2], %	20.3	20.3	20.3	20.3
Crude fiber[2], %	4.3	4.7	4.7	4.7
Fe, *mg/kg*	100.8	94.9	95.7	112.1
Inulin, *g/kg*	7.4	38.4	41.9	44.8

[1]Same vitamin and mineral premix were used as in Expt. 1 of Chapter 2.

[2]Calculated using NRC (1998).

4.4 Results

Whereas all 4 groups of pigs had similar initial blood Hb concentrations at wk 0, final blood Hb concentrations in pigs fed all three types of inulin was (15%, Synergy 1; 20 %, HP; 14%, P95) higher ($P < 0.05$) than pigs fed BD (**Table 2**). Although the final hematocrit of pigs fed HP and P95 were higher ($P < 0.05$) than BD, there was no difference in hematocrit between the BD and Synergy 1 fed pigs. The changes in mean Hb concentrations and total feed intake over the 5-wk period between the treat groups displayed the same statistical outcome as their final blood Hb concentrations (data not shown) and hematocrit (Table 2). Pigs fed 4% Synergy 1 had 12% higher ($P < 0.05$) overall HRE than pigs fed BD or P95 (**Fig. 1**). Pigs fed P95 had 12%, and 28% lower ($P < 0.05$) overall HRE than pigs fed BD and Synergy 1, respectively. The improvement noted for pigs fed HP did not differ from pigs fed the BD or Synergy 1. There was no difference in overall growth performance. Although total iron concentrations of digesta from lower jejunum, ileum, and cecum were not significantly different (**Fig. 2**), pigs fed 4% Synergy 1 in proximal colon, and HP in mid colon had % higher ($P < 0.05$) soluble Fe concentrations than those of pigs fed BD, respectively. On the other hand, pigs fed 4% P95 had lower ($P < 0.05$) soluble Fe concentrations compared to Synergy 1 (51%) in proximal colon, Synergy 1 (42%) and HP (48%) in Mid colon and HP (38%) in distal colon. Digesta soluble sulfide concentration did not differ between the treatment groups (**Fig. 3**). All groups of pigs showed no difference in pH of digesta samples from various segments (**Table 3**), except in stomach where P 95 had lower ($P < 0.05$) pH than the pigs fed HP and in proximal colon where pigs fed HP had lower ($P < 0.05$) than the pigs fed BD and Synergy 1. All groups of pigs

showed no difference in digesta dry matter contents from various segments (**Table 4**), except in the mid colon where P95 fed pigs had 26% higher ($P < 0.05$) dry matter contents than the pigs fed HP.

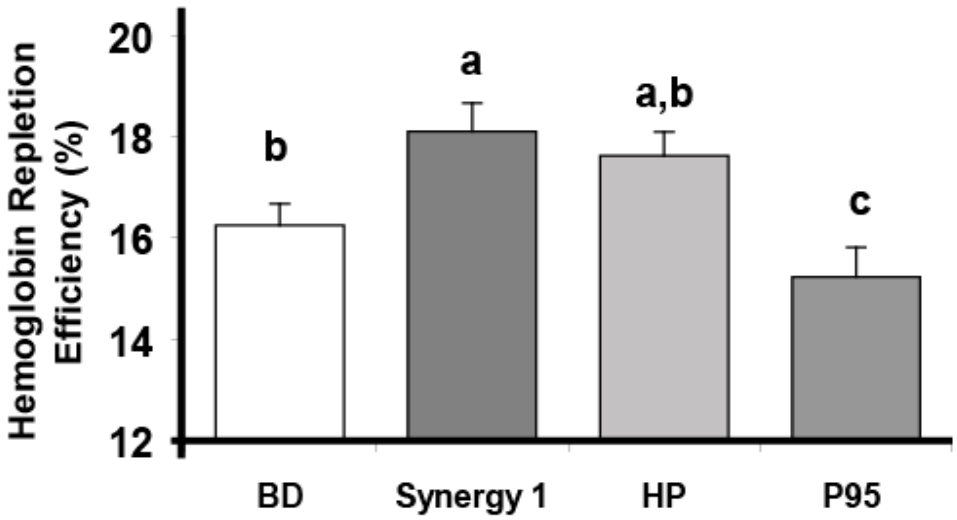

Figure 4.1 Effects of different types of inulin on hemoglobin repletion efficiency (HRE) of pigs. The calculation of HRE is described in the text (85). Values are means ± SEM, $n = 8$. Means without a common letter differ, $P \leq 0.05$.

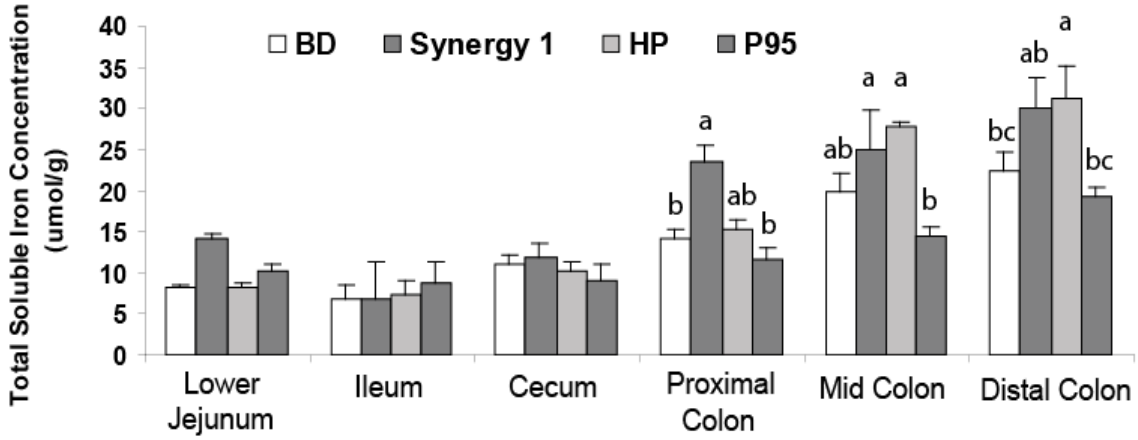

Figure 4.2 Effect of different types of supplemental inulin on total soluble iron concentration of digesta of pigs. Values are means ± SEM, $n = 8$. Means without a common letter differ, $P \leq 0.05$.

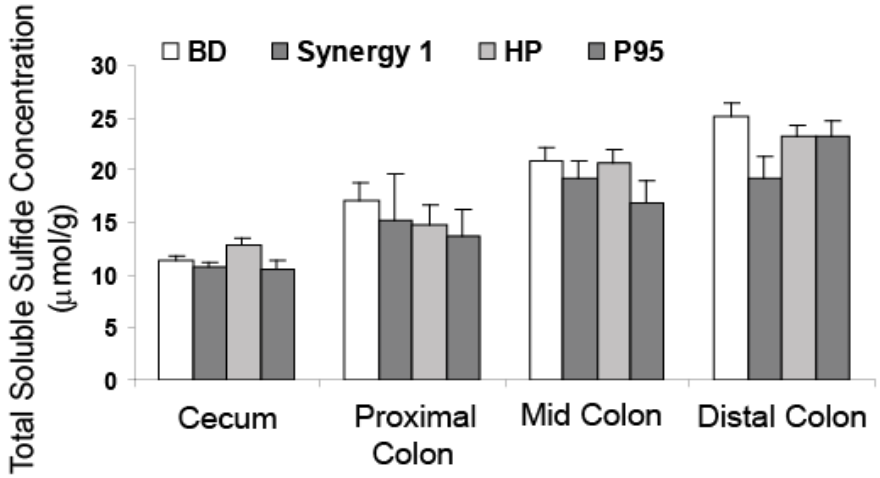

Figure 4.3 Effect of different types of supplemental inulin on total soluble sulfide concentration of digesta of pigs. Data were analyzed using 1-way ANOVA. Values are means ± SEM, $n = 8$.

Table 4.2 Effect of different types of inulin on growth performance, and blood hemoglobin concentration of pigs. Values are means, $n = 8$. Means in a row with superscripts without a common letter differ, $P < 0.05$. Data were analyzed using 1-way ANOVA with (Hb, hematocrit, and body weight) or without (weight gain, and feed intake) time-repeated measurements.

	Time, *wk*	BD	Synergy 1	HP	P95	SEM
Hemoglobin, g/L	0	86.6	83.7	85.0	84.1	1.83
	5	117.8[a]	129.4[b]	128.0[b]	130.0[b]	2.31
Hematocrit, %	0	34.0	34.6	34.3	35.3	1.42
	5	39.1[a]	41.4[a,b]	42.1[b]	42.7[b]	0.95
Body weight, kg	0	9.1	8.8	9.2	9.5	0.41
	5	32.1	32.05	33.7	34.2	1.12
Weight gain, kg/d	0-5	0.65	0.65	0.70	0.70	0.02
Feed intake, g/d	0-5	1118.4[a]	1182.6[a,b]	1310.2[b,c]	1359.2[c]	48.6

Table 4.3 Effect of different types of dietary supplemental inulin on digesta pH of pigs. Values are means ± SEM, $n = 8$. Means without a common letter differ, $P \leq 0.05$.

	BD	Synergy 1	HP	P95	SEM
Stomach	$4.24^{a,b}$	$4.25^{a,b}$	4.50^{b}	3.43^{a}	0.34
Lower Jejunum	7.07	6.70	6.95	6.95	0.23
Ileum	6.87	6.66	6.79	7.01	0.24
Cecum	5.89	5.77	5.74	5.82	0.09
Proximal Colon	6.13^{b}	6.07^{b}	5.71^{a}	$5.87^{a,b}$	0.11
Mid Colon	6.49	6.32	6.51	6.28	0.15
Distal Colon	6.59	6.55	6.58	6.73	0.13

Table 4.4 Effect of different types of dietary supplemental inulin on digesta dry matter contents of pigs. Values are mean, $n = 8$. *Marginally higher ($P < 0.08$) compared to the HP supplemented diet fed pigs.

	BD	Synergy 1	HP	P95	SEM
			%		
Stomach	19.3	19.6	21.9	20.6	1.32
Lower Jejunum	14.5	16.1	14.9	13.5	1.13
Ileum	13.3	12.8	14.2	12.4	1.15
Cecum	14.9	14.8	14.8	15.1	0.57
Proximal Colon	17.4	17.8	17.3	18.8	1.22
Mid Colon	24.4	22.8	21.5	25.3*	1.50
Distal Colon	26.2	28.5	22.9	28.5	2.32

4.5 Discussion

All three types of supplemental inulin added to corn-soybean meal diets significantly increased hemoglobin concentrations in weanling pigs. Previously, we showed such positive effects in pigs fed inulin that was composed of 50:50 mixture of long and short-chain inulin (Synergy 1) however the present study is the first time to show such positive effects on pigs fed long (HP) and short-chain (P95) inulin. These findings are extremely encouraging and further support the use of inulin-type fructans in diets as a strategy for improving human iron nutrition.

In the present study, the positive effect of inulin on hemoglobin concentrations was again associated with hemoglobin repletion efficiency (HRE) when Synergy 1 was consumed by pigs (4). However, such improvement in hemoglobin concentrations were not associated, and negatively associated with HRE when pigs were fed long (HP) and short-chain (P95) inulin, respectively. This suggests that the positive effects on hemoglobin concentrations by the supplementation of inulin are not always caused by the improvement of HRE. Presumably, different chain-length inulin may improve hemoglobin concentrations through different mechanisms.

Interestingly, HP and P95 fed pigs had higher daily feed intakes compared to BD fed pigs. Consequently, increasing feed intake would enhance the intake of iron. This presumably, makes more iron available in the digestive tract for absorption. Determining the cause(s) of such enhanced feed intake in pigs fed HP and P95 is difficult. Non-digestible carbohydrate is known to have a low digestibility, which increases feed intake of animals due to their reduced nutritional values (126). However, Synergy 1, which also is a non-digestible carbohydrate did not show such enhanced feed intake. It is also interesting to note that shorter the fructose units of inulin, they taste rather sweeter (120). Presumably, the fact that P95 fed pigs had the highest feed

intake may be explained by the sweetness of the diet caused by the supplementation of P95 which may have encouraged the pigs to consume more.

Our data on digesta soluble iron and sulfide concentrations was not in agreement with our previous study (4). We showed previously significantly higher soluble iron concentrations in all three segments of the colon and lowered sulfide concentration in the distal colon of pigs fed Synergy 1. In the present study, however, we did not see such clear increase in soluble iron and any differences in soluble sulfide in all three types of inulin. Because we did not determine the total iron concentrations of digesta in the present study, it is difficult to assess whether such changes is due to the differences in the total digesta iron content or the soluble portion of the total iron.

In conclusion, our results indicate that supplementation of all three types of inulin improved hemoglobin concentrations. The positive effect of inulin on hemoglobin concentrations was not always associated with enhanced HRE or the concentrations of digesta soluble iron and sulfide. Our results suggest that different types of inulin may improve the iron status of animals through different mechanisms.

CHAPTER FIVE

Summary

The overall objectives of this thesis were to 1) determine whether supplementation of inulin affect the bioavailability of iron and 2) to illustrate the metabolic mechanisms for that improvement conferred by inulin, and 3) to characterize the site(s) of inulin degradation in the G.I. tract of pigs. One type of inulin (50:50 mixture of long and short chain inulin) have shown to enhance the bioavailability of iron intrinsically present in corn and soybean meal by pigs when 4% of that inulin was supplemented (See Chapter 2 and 4). In addition, all three types of inulin tested ((1)long and (2)short chain inulin and (3)mixture of long and short chain inulin) increased hemoglobin concentrations in weanling pigs. However, such positive effects were not always mediated by the improvement of HRE. Therefore, we can speculate that different chain-length inulin may improve hemoglobin concentrations through different mechanisms.

Evidence on the sites of inulin degradation in the gastrointestinal tract (G.I.) of humans and pigs is conflicting. Comparisons of inulin concentrations between feces and effluents of the ileostomy patients indicate virtually no degradation or absorption of inulin proximal to large intestines of humans. In contrast, > 90% of ingested inulin is digested pre-caecally in pigs and argued against pigs as a proper animal model for humans in this regard. However these pig studies had major technical limitations for precisely identifying the location of inulin degration. Therefore, we used more direct and sensitive methods to determine the site(s) of inulin degradation in the G.I. tract. Our results suggested that the cecum is the major degradation site of ingested inulin in the G.I. tract of young pigs. Our finding helped clarify the confusion created by previous experiments with technical drawbacks that pigs digest inulin and

oligosaccharides nearly completely proximal to the cecum, and supports the continuous application of pigs as a model of humans for inulin studies.

Further research into the mechanism of how different types of inulin improving the bioavailability of iron in pigs are justified. Understanding such mechanism would allow us to maximize positive effects of inulin on iron nutrition and other claimed health beneficial effects in humans.

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