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Iron absorption and regulatory mechanisms: effects of fructooligosaccharide and other prebiotics

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Iron Absorption and Regulatory Mechanisms: Effects of Fructooligosaccharide and Other Prebiotics

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A thesis submitted in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

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July 2017

DECLARATION

I hereby declare that this thesis represents my own work which has been done after registration for the degree of PhD at Hong Kong Baptist University, and has not been previously included in a thesis or dissertation submitted to this or any other institution for a degree, diploma or other qualifications.

I have read the University's current research ethics guidelines, and accept responsibility for the conduct of the procedures in accordance with the University's Committee on the Use of Human & Animal Subjects in Teaching and Research (HASC). I have attempted to identify all the risks related to this research that may arise in conducting this research, obtained the relevant ethical and/or safety approval (where applicable), and acknowledged my obligations and rights of the participants.

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Abstract

Iron deficiency is the most prevalent nutrient deficiency in the world, leading to long-term developmental and health consequences in populations at risk. Also known as prebiotics, non-digestible oligosaccharides such as fructooligosaccharide (FOS), inulin, galactooligosaccharide (GOS) and lactulose resist digestion by gastric acid and pancreatic enzymes *in vivo*, but are preferentially fermented by beneficial intestinal bacteria once they reach the colon. Prebiotics have been shown to increase the absorption of minerals such as iron from diets, but results from studies reported in the literature at times are contradictory, and mechanisms involved are still unclear. A better understanding of the role of FOS and other prebiotics in iron absorption may lead to new dietary modification strategies to increase intake of iron absorption enhancers in plant-based diets. The objectives of this study were therefore to determine the effects of prolonged FOS, as well as Synergy 1 (a combination of long- and short-chain FOS), inulin, GOS and lactulose supplementation on iron status of anemic rats; and to assess the enhancing effects of FOS on iron absorption and elucidate the regulatory mechanism involved using the Caco-2 cell culture model.

In our animal studies, male Sprague-Dawley rats were first fed a low-iron diet for 14 days prior to prebiotics supplementation to achieve an iron-deficient status. Rats receiving the low-iron diet (12 ppm Fe) showed significantly lower non-heme iron concentrations in liver, spleen and kidney, as well as lower hemoglobin level than rats receiving a normal diet (45 ppm Fe), confirming iron-deficiency anemia.

At the onset of the feeding trials, anemic rats were further divided into groups with or without supplementation of prebiotics. Prebiotics were provided to the rats by dissolving in water at 5% (w/v). Rats were kept on their respective test diets for 28 or 35 days, and all had free access to food and water during the feeding trials. The results showed significantly higher hemoglobin and non-heme iron levels in anemic rats with FOS or GOS supplementation, suggesting that both FOS and GOS could have positive effects on the iron status of anemic subjects with a low-iron intake. Rat colon contents also showed significant changes in short-chain fatty acid (SCFA) concentrations, presumably due to fermentation of prebiotics by intestinal microflora.

Changes in the expression of Duodenal cytochrome b (Dcytb) and Divalent metal transporter 1 (DMT-1) in Caco-2 cells were measured by Western Blot and Real Time PCR. Our results confirmed that Caco-2 cells 14 days post confluence provided a stable research model for gene expression studies related to iron absorption. At low iron level, especially with FOS or SCFA supplementation, Dcytb and DMT-1 expression levels were increased in Caco-2 cells. While at high iron level, expression of Dcytb or DMT-1 was mostly down-regulated. Effects of SCFA were much more pronounced than FOS at different iron concentrations, suggesting that any effects of dietary FOS on improving iron status would require fermentation by the intestinal microflora. Further studies on other prebiotics (e.g., GOS and lactulose) and different combinations of SCFA are warranted.

Key words: iron, anemia, FOS, Caco-2, Dcytb, DMT-1

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List of Abbreviations and Acronyms

BMP	Bone morphogenetic protein
Cp	Ceruloplasmin
Dcytb	Duodenal cytochrome b
DMT-1	Divalent metal transporter 1
FOS	Fructooligosaccharide
GOS	Galactooligosaccharide
IREG1	Ferroportin 1
HAMP	Hepcidin antimicrobial peptide
HCP1	Heme carrier protein 1
HFE	Haemochromatosis protein
HIF	Hypoxia-inducible factor
HJV	Hemojuvelin
HREs	HIF responsive elements
ID	Iron deficiency
IDA	Iron-deficiency anemia
IRE	Iron-responsive element
IRPs	Iron regulatory proteins
KO	Knockout
PCBP1	Poly (rC)-binding protein 1
PCFT	Proton-coupled folate transporter
PHD	Prolyl hydroxylases

SCFA	Short-chain fatty acid
Tf	Transferrin
TfR1	Transferrin receptor 1
UTR	Untranslated region
w/	With
w/o or w/t	Without

Chapter 1 General Introduction

1.1 General introduction of iron

Iron is an essential trace element in the human body. It exists in body cells and serves many vital functions. The essentiality of iron was confirmed in 1932 when the need for inorganic iron for hemoglobin synthesis was proven (Yip and Dallman, 1996). In humans, iron is required for many enzymes and proteins involved in biological reactions or in transport systems. For instance, iron as part of the hemoglobin and myoglobin, could carry and store oxygen in blood and muscle, respectively. In addition, it is the required co-factor (active site) for many important redox enzymes in numerous metabolic reactions which are critical in maintaining normal physiological functions (McDowell, 2003). Iron is also the transport medium for electrons when it is the co-factor of cytochrome (McDowell, 2003; Hurrell, 1997).

Iron requirement is different for different age and sex groups. As shown in Table 1.1, a daily iron intake is needed for the replacement of iron loss in the human body. The need for iron for infants increases greatly at 4-12 months after birth. Adolescents, particularly during the period of growth also require higher amount of iron. The population groups with the highest demand for iron are pregnant women during the second and third trimesters (6.3 mg/day), followed by 12-16 years' girls (2.02 mg/day), and menstruating women (2.38 mg/day).

Table 1.1 Iron requirements of 97.5% of individuals in terms of absorbed iron, by age and sex (WHO)

Age/sex	mg/day
4-12 months	0.96
13-24 months	0.61
2-5 years	0.70
6-11 years	1.17
12-16 years (girls)	2.02
12-16 years (boys)	1.82
Adult males	1.14
Pregnant women	
First trimester	0.80
Second and third trimester	6.30
Lactating women	1.31
Menstruating women	2.38
Postmenopausal women	0.96

Nutritional iron deficiency (ID) occurs when physiological requirements could not be met by iron uptake from the diet (Larocque et al., 2005). People at the life stages of high iron demand are at a higher risk of developing ID if they don't have adequate access to foods rich in bioavailable iron. Thus, young children and adolescents due to periods of rapid growth, as well as women of reproductive age, especially during pregnancy are most at risk. For women of reproductive age, more iron is needed because of the excess loss of blood during menstruation, and during pregnancy, the rapid growth of the placenta and fetus lead to a greater demand of iron (Dallman, 1990).

Diets chronically low in bioavailable iron could lead to long-term developmental and health consequences, and could even lead to higher mortality

rate (Yip, 1994). ID is one of the most prevalent diet-related nutritional deficiencies in the world, and it remains a concern in both developing and developed countries (Cook et al., 1976; Sikosana et al., 1998). At its most severe stage, ID could lead to iron-deficiency anemia (IDA), which is a major public health issue affecting up to 1 billion people. (Abbaspour et al., 2014). IDA, even in mild and moderate forms, can cause functional impairments, affecting cognitive development and learning ability (Beard and Connor, 2003), immunity mechanisms (Failla, 2003), work capacity (Viteri and Torun, 1974), and rate of morbidity (CDC, 2010). During pregnancy, ID is associated with various adverse consequences for both mother and infant, including increased risk of severe anemia and sepsis, higher maternal morbidity and mortality (Khan et al., 2006), perinatal mortality and low birth weight (CDC, 2010), and a higher risk of preterm delivery in early pregnancy (Scholl, 2005).

Although the scientific basis of food iron absorption is relatively well understood, it has been estimated that 1/3 of the global population is iron deficient (Beard and Stoltzfus, 2001; Gillespie, 1998). Both ID and IDA are prevalent in developing countries, especially for infants, young children and women of childbearing age (Looker et al., 1997; Lynch, 2011; Cogswell, 2009; Zimmermann and Hurrell, 2007), presumably because their diets could not provide sufficient bioavailable iron to meet their needs for growth, pregnancy, and replacement of menstrual losses (Lynch, 2011). Moreover, even in developed countries such as the United States, 9%-11% of young children and women of childbearing age are iron deficient, and up to 5% of them are anemic (Looker et al., 1997). ID is also one of

the leading risk factors that causes disability and death (Zimmermann and Hurrell, 2007). As a result, reducing the prevalence of ID has remained a priority for WHO since 1992.

In general, either a decrease in iron absorption or the amount of iron intake, or an increase in the need for iron by the body could lead to ID (Stöppler, 2013). Nevertheless, poor bioavailability of dietary iron has been a major factor contributing to ID (Hurrell, 1997; Zimmermann et al., 2005), and is the main underlying cause for most anemia in developed countries (WHO, 2006). Iron absorption rate may range from 5% to 35%, depending on the type of iron as well as the presence of other dietary factors (McDowell, 2003). Dietary modifications to improve the bioavailability of iron could be possible strategies for combating ID (Stöppler, 2013).

1.2 Intestinal iron absorption

The small intestine plays a key role in iron absorption, as iron is predominantly absorbed through the brush border membrane of the enterocyte in the proximal duodenum and upper jejunum, with the villi and microvilli present on the intestinal lumen surface helping to maximize the area of absorption (Muir and Hopfer, 1985). In general, there are three steps in iron absorption: first, iron is taken from the intestinal lumen into the enterocyte; second, iron goes through intracellular metabolism and transport; and third, iron is exported through the basolateral membrane of the enterocyte into the blood for circulation. The control of iron homeostasis is of importance, as ID causes IDA and iron overload leads to hemochromatosis. As there is no pathway for iron loss regulation, the hemostasis of body iron requires tightly modulated mechanisms for regulating iron absorption and mobilization from stores in order to provide adequate, but not overabundant, iron to satisfy body iron requirement and reserve (Hentze et al., 2004). The duodenum itself is not only an important sensor but also an important regulator of iron absorption. According to the crypt cell hypothesis, the mucosa of duodenum is believed to be a key sensor for iron uptake since iron absorption related proteins are highly expressed in mature enterocytes which are differentiated by crypt stem cells after migrated to the villi (Simpson and McKie, 2009). Besides, it also responds to systemic iron needs by changing its iron content and altering protein expression. The mucosa control of iron absorption had been reported by Mastrogiannaki et al. (2009) and Shah et al. (2009) that duodenal mucosa regulated iron absorption

related protein expression, especially Duodenal cytochrome b (Dcytb) and Divalent metal transporter 1 (DMT-1) by the transcription factor hypoxia-inducible factor-2α (HIF-2α). Iron taken up by enterocytes in the crypt region of the duodenal mucosa from plasma is proportional to the body's iron level, and the body iron status is reflected by intracellular iron level (Oates et al., 2000a, 2000b).

It should be noted that there are two forms of dietary iron: heme and non-heme iron, and absorption of these two forms of iron is regulated by different mechanisms in terms of the first two steps (Zimmermann et al., 2007; Trinder et al., 2002). Each form of the dietary iron has its specific transporters at the brush border.

For heme iron, it is suggested that apical heme is taken up into the enterocyte as an intact metalloporphyrin by endocytosis. Heme carrier protein 1 (HCP1)/ proton-coupled folate transporter (PCFT), first described by Shayeghi et al (2005), is a putative intestinal heme iron transporter, which might also transport folate. It is up-regulated by hypoxia and ID (Shayeghi et al., 2005; Qiu et al., 2006). Once inside the enterocyte, ferrous iron can be released from the heme complex by heme oxygenase and joins the intracellular iron pool, which later could be either exported across the basolateral membrane into the blood by ferroportin or incorporated into ferritin for transient storage (Trinder et al., 2002). The process of heme iron absorption is much more efficient than non-heme iron absorption and is less susceptible to duodenal pH and dietary factors (Abbaspour et al., 2014).

For non-heme iron that exists primarily in less soluble ferric form, it has to be reduced to ferrous form first at the brush border, and then absorbed through a

ferrous iron transporter. Several proteins relevant to the non-heme iron absorption process have been reported.

Duodenal cytochrome b (Dcytb), also known as Cybrd1, is a ferrireductase in mammals and first identified by Mckie et al. (2001). It is a membrane protein with six transmembrane domains and is homologous to cytochrome b561. Dcytb is highly expressed in the apical membrane of duodenal microvilli and it has been suggested to play a key role in non-heme iron absorption. Using an iron-deficient rat model, Dcytb has been shown with a subtractive cloning strategy to be rapidly regulated by ID, hypoxia and increased systemic iron requirements (McKie et al., 2001; McKie, 2008). However, Gunshin et al. (2005) showed that Dcytb-knockout mice fed with a normal iron diet did not develop ID. The lack of effects in Dcytb-knockout mice suggested that there might be other supplementary ferrireductases or ferrireductants such as ascorbic acid (Zimmermann et al., 2007; Atanasova et al., 2004). Moreover, the standard diet used by Gunshin et al. (2005) contained a high amount of ferrous iron, and thus the need for ferrireductase activity of Dcytb might be minimized, as there is no need for ferric iron to be reduced to ferrous form (Frazer et al., 2005). Nevertheless, the spleen non-heme iron level in Dcytb-knockout mice has been shown to be half of the wildtype (Choi et al., 2012), suggesting a role for Dcytb in maintaining iron status. McKie et al. (2001) also found that Dcytb was likely to be the only hypoxia-inducible ferrireductase in duodenum as Dcytb-knockout mice showed no hypoxia-inducible ferrireductase activity. Taken together, Dcytb plays a critical role in non-heme iron

absorption and systemic iron metabolism and is the primary duodenal ferrireductase.

Divalent metal transporter 1 (DMT-1), also known as solute carrier 11 family A2 (SLC11A2), natural resistance-associated macrophage protein 2 (Nramp2) or divalent cation transporter 1 (DCT-1), is a proton-coupled transporter that mediates non-heme iron from intestinal lumen into the enterocyte after the reduction of ferric iron (Gunshin et al., 1997). It transports ferrous iron and also a number of other divalent metal ions including copper, cobalt, zinc, and lead (Gunshin et al., 1997; Yeung et al., 2005b). DMT-1 is widely expressed in body cells but has the highest expression on apical membrane of duodenal enterocytes; and similar to Dcytb, its expression is influenced by iron status. There are 17 exons in human DMT-1 gene encoding four splice variants with equivalent function. DMT-1 isoform 1A is from the mRNA starting from exon 1A at 5'-end, while isoform 1B is from the one starting from exon 1B at 5'-end. Besides, the alternative splicing at 3'-UTR leads to different splice variants with or without 3'-end iron-responsive element (+IRE/-IRE) respectively (Mackenzie et al. 2007). Expression and subcellular distribution of DMT-1 isoforms are different. Isoform 1A is tissue-specific and is predominantly found in the duodenum and kidney, whereas 1B is ubiquitously expressed. Isoform with IRE (+IRE) is mainly expressed at epithelial plasma membrane for apical iron uptake and responds to iron status, while isoform without IRE is non-specific and is expressed in many cells (Mackenzie et al. 2007). DMT-1 has been studied in both animals and human cell cultures, and both isoform 1A and

isoform +IRE are responsible for regulating iron status (Mackenzie et al. 2007). Mice with DTM-1 gene knockout in intestine are associated with anemia. Mutation in DMT-1 in animals, such as Gly-185-Arg mutation in DMT-1 gene in Microcytic anemia (mk) mice and Belgrade (b) rats, caused systemic ID and microcytic anemia, suggesting an important role of DMT-1 in intestinal ferrous non-heme iron uptake and transport (Fleming et al., 1998; Fleming et al., 1997).

Once inside the enterocyte, some iron is then transiently stored in an iron storage protein, namely ferritin, for future use or export; some is utilized for heme or other functional proteins' synthesis; and the remaining iron is transferred out across the basolateral membrane into the blood for circulation by a membrane bound protein called ferroportin 1 (also known as IREG1, FPN-1 and MTP1), in association with an iron oxidase hephaestin (Donovan et al., 2000; Donovan et al., 2005; Mckie et al., 2000; Abboud et al., 2000). Extracellular ferrous iron is bound by plasma transferrin (Tf) after oxidation by hephaestin (Vulpe et al., 1999). (See Fig. 1.1.) Transferrin receptor 1 (TfR1), which is expressed in crypt cells, is a protein that mediates the uptake of transferrin bound iron (Anderson et al., 1990). A highly expressed protein in crypt cells called haemochromatosis protein (HFE) regulates the TfR1 mediated transferrin bound iron uptake (Parkkila et al., 1997; Waheed et al., 1999).

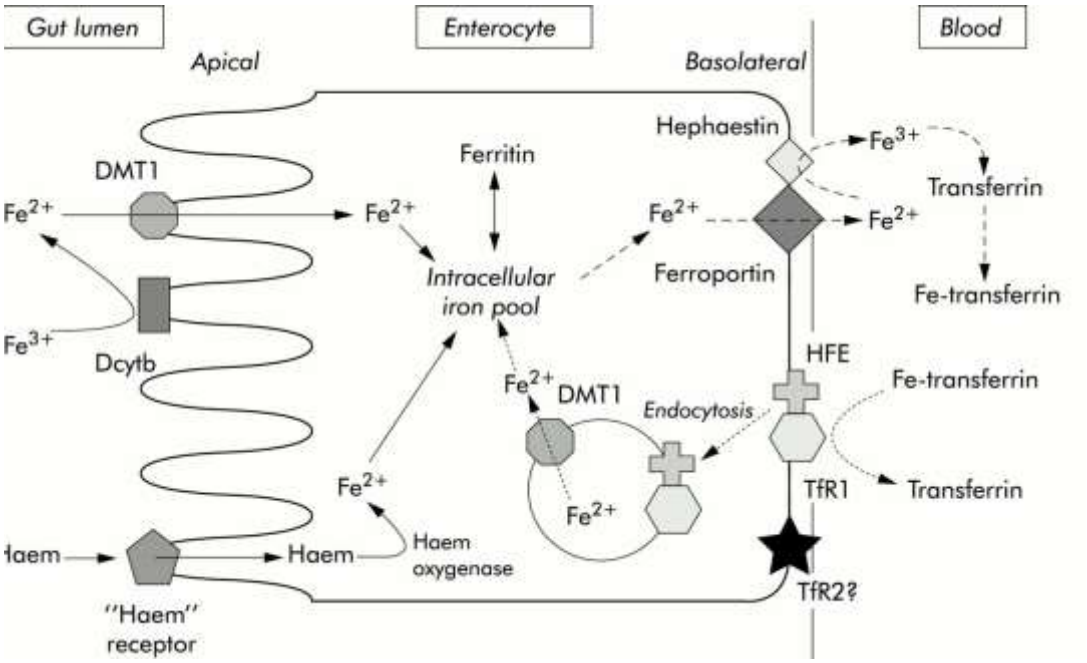


Fig. 1.1 A model of the pathways of iron absorption by the enterocyte (Trinder et al., 2002)

1.3 Mechanism and regulation of intestinal iron absorption and dietary factors

As a defined mechanism for the active excretion of iron is lacking in the body, the mechanism of regulation of intestinal iron absorption should therefore be the main focus for iron homeostasis regulation (Hurrell and Egli, 2010; Finberg, 2011). The absorption mainly occurs in the duodenum, where villi and microvilli could maximize the absorption area. As mentioned above, dietary ferric iron would be reduced by Dcytb before taken up by DMT-1 into duodenal enterocytes. Cytosol ferrous iron can be stored in cell or be exported into blood for circulation by IREG1 and subsequently oxidized by hephaestin, bound to Tf in circulation and delivered to tissues.

Ferric iron is not soluble at pH > 3, and physiological pH of duodenum ranges from 4 to 7. Therefore, ferric iron is insoluble in the natural pH environment of the duodenum and may not be absorbed, whereas ferrous iron is soluble even up to pH 7.5 (Ma, 2012). Ferric iron can be greatly reduced by gastric H^+ or dietary ascorbic acid in stomach, and in duodenum, ascorbic acid could reduce ferric iron by forming soluble complexes, and thus increase iron absorption. The enhancing effect of ascorbic acid was confirmed when iron deficiency in rats resulted in an induction of gene encoding sodium-dependent vitamin C transporter (SVCT1) and an increase in enterocyte ascorbate level (Collins et al. 2005). In addition, the effect of Dcytb can also enhanced by ascorbic acid, which may serve as an intracellular electron donor to facilitate iron reduction for transport (Latunde-Dada et al. 2008).

Mutation of DMT-1 in human could lead to iron overload (Mims et al. 2005; Beaumont et al. 2006) because DMT-1 is ubiquitously expressed and the mutation does not completely suppress its function in duodenum, but affects its function in other tissues. When there are ID signals in body tissues, iron absorption, including heme iron absorption would be increased, but utilization would be decreased because of an impaired Tf cycle due to the suppressed function of DMT-1, resulting in iron overload (Mims et al. 2005; Beaumont et al. 2006).

When iron enters the enterocyte, there are 3 possible ways for metabolism. Some will be used for heme synthesis, iron-sulfur cluster and cell functional proteins; some will be stored in ferritin and some will enter into circulation. Degradation of ferritin would help iron mobilization for usage or exportation via IREG1, and expression of IREG1 could in return mediate iron mobilization from ferritin degradation by proteasomes. In the presence of iron chelator desferrioxamine or when autophagy happens, ferritin would be degraded in lysosome (De Domenico et al. 2006). The subsequent intracellular transfer of ferrous iron may be related to iron chaperones, such as frataxin, which is an iron chaperone in mitochondria (Bulteau et al. 2004). Besides, loading of iron to ferritin could be facilitated by human poly (rC)-binding protein 1 (PCBP1) in cytosol, and depletion of PCBP1 could inhibit ferritin formation and increase intracellular iron pool (Shi et al. 2008).

The basolateral iron export needs not only iron transporter protein but also ferroxidase. IREG1 is the only iron exporter in mammals and is highly expressed in

iron-deficient intestine. It mediates iron export to circulation in cooperation with hephaestin, which is homologous to ceruloplasmin (Cp), a plasma ferroxidase (Donovan et al. 2000). Hephaestin facilitates the iron export, and both hephaestin and Cp could oxidize ferrous iron to ferric iron, allowing iron to bind to serum Tf. Han et al. (2007) showed that hephaestin and IREG1 cooperate together for iron exportation. And De Domenico et al. (2007a) reported that Cp is required to maintain the localization of IREG1 on cell surface.

Imbalances of systemic iron from either ID or iron overload results in some of the most common diseases in humans, including IDA and hemochromatosis. On one hand, intestinal iron absorption can be regulated at the post-translational, transcriptional and post-transcriptional levels by different regulatory factors: hepcidin, hypoxia-inducible factor-2α (HIF-2α) and IRE/IRP systems respectively, according to pathophysiological conditions, such as systemic iron levels and hypoxia. On the other hand, intestinal iron absorption can be influenced by dietary factors at the absorption level.

Hepcidin is a liver-derived circulating peptide hormone regulator for systemic iron homeostasis. It has been shown to regulate iron fluxes in the body and possess antimicrobial activity *in vitro* (Peslova et al. 2009). It is secreted by hepatocytes and acts on small intestine, macrophages and liver. The action of hepcidin is a post-translational modification of IREG1 to reduce the iron release into plasma (Peslova et al. 2009; Nemeth et al. 2004; De Domenico et al. 2007a). Hepcidin is bound by α2-macroglobulin and circulates in plasma. When signal appears, it acts

by binding to IREG1 (which helps to transfer iron out of the basolateral membrane into the blood) on the cell surface of target tissues and serves as a negative regulator, causing IREG1 internalization and subsequent ubiquitination, and lysosomal degradation (Peslova et al. 2009; Nemeth et al., 2004), i.e., high level of hepcidin can cause the loss of IREG1 from the cell surface and prevent iron from getting into the blood. Conversely, decreased expression of hepcidin could result in increased IREG1 and increased plasma iron. Plasma hepcidin can be cleared by kidney filtration or internalization with IREG1 into target cells, neither of which is a regulated process. The control of hepcidin is its production in liver at the transcriptional level when response to pathophysiological iron signals (De Domenico et al., 2007b). Impaired expression of hepcidin has been shown to be due to the mutations in any of the following genes: transferrin receptor 2 (TfR2), hemochromatosis (HFE), hemochromatosis type 2 (HFE2) (also known as hemojuvelin, HJV) and hepcidin antimicrobial peptide (HAMP). The plasma hepcidin level could be regulated by cytokines, plasma iron, anemia and hypoxia (De Domenico et al., 2007b).

As to the response to systemic iron levels, two signaling pathways for liver hepcidin regulation have been well characterized: TfR1-HFE-TfR2 signaling and BMP-HJV signaling pathways, which are responses to the plasma Tf-Fe_2 level and to iron status by bone morphogenetic protein 6 (BMP6), respectively (Goswami et al. 2006). In TfR1-HFE-TfR2 signaling pathway, the expression of TfR1 is ubiquitous, while expression of TfR2 is high in liver and TfR2 has lower binding

affinity to HFE than TfR1. HFE competes for the binding site on TfR1 with plasma Tf-Fe_2 and it works as a switch between TfR1 and TfR2 (Goswami et al. 2006). When a high level of plasma Tf-Fe_2 is present, more Tf-Fe_2 would bind to TfR1 and replace HFE from TfR1, which would promote the binding of HFE to TfR2 and in turn triggers a signal transduction, inducing hepcidin transcription and subsequent reduction of iron release into plasma and therefore reduction of plasma Tf-Fe_2 levels (Poli et al. 2010; Gao et al. 2009). Overall, high level TfR1-HFE binding causes low hepcidin expression and lead to iron overload, and more TfR2-HFE binding results in high level of hepcidin and then causes iron deficiency (Wallace et al. 2007; Schmidt et al. 2008). BMP-HJV (BMP-HJV-SMAD) signaling pathway is critical in hepcidin production regulation and HJV is the key for hepcidin expression. HJV is a co-receptor of BMP on cell surface (Babitt et al. 2006). It forms complex with BMP (BMP/HJV) and conjugates to BMP receptors to induce downstream signals, including SMAD4 (liver-specific sole mammalian co-SMAD factor) activation, which then induces hepcidin transcription (Wang et al. 2005). BMP6 is positively regulated by iron status, while other BMPs are opposite (Meynard et al. 2009). The stabilization of HJV on cell surface would maintain the BMP signaling and induce hepcidin transcription, therefore reduce plasma iron level. In addition, HJV can be cleaved off from the cell membrane and released as soluble HJV at low iron status (Lin et al. 2005). The soluble HJV could lower hepcidin expression and elevate iron release into plasma.

Hepcidin expression can also be regulated by erythropoietic signals. Its expression is repressed in the condition of IDA, hemolytic anemia and anemia with ineffective erythropoiesis (Pak et al. 2006). Two erythropoietic signals, growth differentiation factor (GDF15) and twisted gastrulation protein (TWSG1), had been reported to suppress hepcidin expression during anemia (Tanno et al. 2007; Tanno et al. 2009). Hypoxia is another factor that can regulate hepcidin expression. The production of hepcidin is reduced at hypoxia condition, by direct regulation of HIFs factors (Peyssonnaux et al. 2007) or indirect modulation of the HJV-BMP signaling pathway (Silvestri et al. 2008; Lakhal et al. 2011) or erythropoietic signals (Robach et al. 2009).

Another regulatory factor for iron absorption is HIF, which was originally discovered as the major oxygen-regulated transcription factor. There are several different HIF α isoforms (HIF-1α, HIF-2α, HIF-3α) and prolyl hydroxylases (PHD) isoforms (PHD1, PHD2, PHD3), which are tissue specific (Epstein et al. 2001). HIF-2α has been reported to induce Dcytb and DMT-1 expression drastically, and induce IREG1 expression to a certain degree (Shah et al. 2009; Mastrogiannaki et al. 2009). PHDs are required for HIF subunit modification in the presence of oxygen, iron or ascorbate (Epstein et al. 2001). HIF can bind to the promoter of hepcidin and repress hepcidin expression. Besides, HIF-2α can control the iron utilization in mitochondria by regulating mitochondrial aconitase chaperone protein (Oktay et al. 2007). If oxygen, iron or ascorbate is present, a subunit of HIF would be modified by PHDs and then HIF could interact with ubiquitin ligase and be degraded

subsequently by proteasome. However, when under the condition of hypoxia or iron deficiency, PHDs' activity would be inhibited, results in accumulation and translocation of HIF to nucleus (Epstein et al. 2001). Therefore, the expression of HIF is negatively regulated by iron and oxygen and HIF is a transcription factor for iron regulation. In addition to PHDs, HIF-2α is important in the regulation of iron transport proteins in duodenum, specifically for Dcytb and DMT-1. It had been shown that Dcytb and DMT-1 were regulated by HIF-2α at transcriptional level (Shah et al. 2009; Mastrogiannaki et al. 2009). In the study by Shah et al. (2009), intestinal-specific VHL knockout (VHL KO) mice (blocking HIF degradation) and VHL/Arnt knockout (VHL/Arnt KO) mice were used (Arnt is required for functional HIF-1α and HIF-2α transcription complexes). In VHL KO mice, mRNA expression of HIF-2α, Dcytb and DMT-1 had been markedly induced by ID, but iron overload was achieved; while in VHL/Arnt KO mice, the responses from Dcytb and DMT-1 genes were lacking under ID and anemia was achieved. In the study by Mastrogiannaki et al. (2009), HIF-1α and HIF-2α had been selectively and directly deleted. HIF-1α KO showed no influence on iron metabolism, while HIF-2α deletion presented noticeable reductions in the mRNA of Dcytb, DMT-1, IREG1 and hepcidin, and decreases in serum iron, iron store and hemoglobin in mice even with normal diet. These studies together suggest that in spite of systemic regulation by hepcidin, the HIF-2α might play a more important role on the control of iron absorption at the apical side by changing Dcytb and DMT-1 expression than on basolateral iron transport (IREG1 and hephaestin).

A third regulatory factor for iron absorption is the IRE/IRP system, which is a post-transcriptional regulation factor. The IRPs include IRP1 and IRP2, and their activities are responsive to the plasma iron fluctuations. When there are changes in plasma Tf-bound iron, IRP activity will be regulated and subsequently iron absorption related proteins will be modulated (Muckenthaler et al. 2008). In ID, the IRPs will be activated and then bind to IREs in untranslated regions (UTRs) of target genes' mRNA. Ferritin (iron store) and iron utilization in enterocytes will decrease, apical iron absorption by DMT-1 will increase, and total iron absorption into blood for circulation will increase as a result. The IRP-IRE binding of the mRNA of ferritin, IREG1, HIF-2α at 5'-UTR will block the translation, while the binding of mRNA of TfR1 and DMT-1 at 3'-UTR will maintain the stability. The IRP binding of IREG1 to block translation in ID may contradict that observation that iron absorption is increased in ID. This might be explained by the study of Zhang et al. (2009) that there is alternative splicing in IREG 1 mRNA, causing some of the mRNA to miss the IRE and thus not regulated by IRP/IRE system. If iron is sufficient, IRP1 will be converted to cytosolic enzyme and IRP2 will be degraded via ubiquitination/proteasome pathway (Salahudeen et al. 2009; Vashisht et al. 2009).

Overall, it has been well established that iron deficiency up-regulates duodenal expression of DMT-1, Dcytb, and ferroportin, and down-regulates the storage protein, ferritin, and thus increasing iron absorption (Mckie et al., 2001;

Canonne-Hergaux et al., 1999; Trinder et al., 2000; Pietrangelo et al., 1992; Collins et al., 2005).

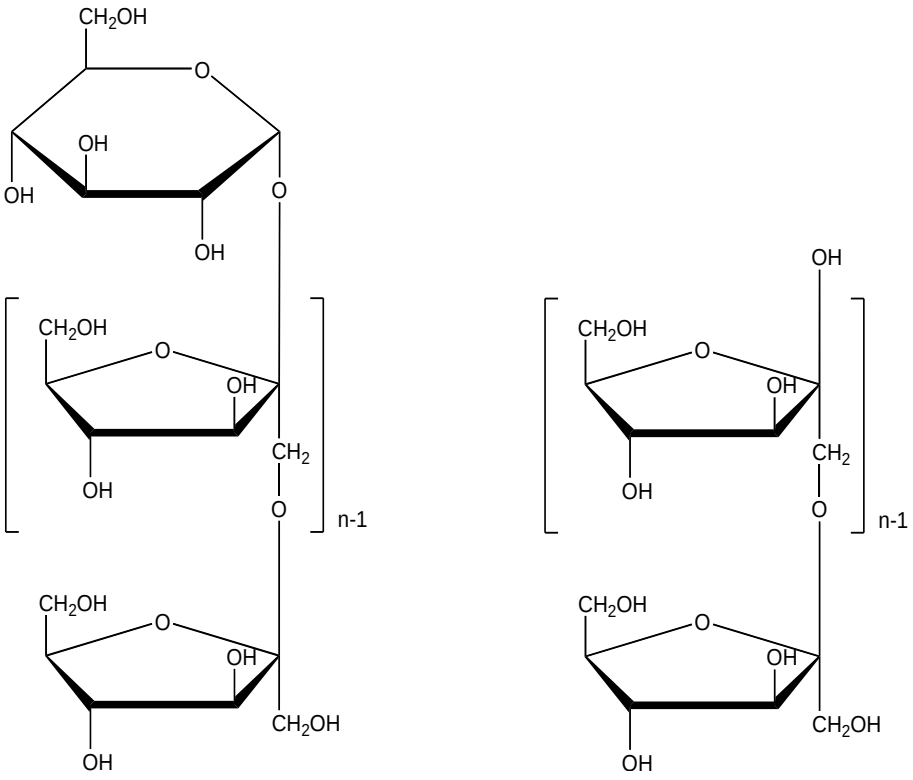
In addition to the regulatory factors mentioned above, intestinal iron absorption can also be affected by dietary factors. The amount of iron absorbed in humans will be determined by the contents of heme and non-heme iron in the diet as well as the balance of dietary factors that influence non-heme iron bioavailability (Brune et al., 1992; Hallberg and Hulthén, 2000). The sources of heme iron are hemoglobin and myoglobin from meat, poultry and fish, while non-heme iron is mainly from plant foods, such as cereals, legumes, fruits and vegetables (FAO/WHO, 2001). Heme iron is relatively more bioavailable than non-heme iron and its absorption is less affected by dietary factors. Iron present in meat is 30–70% heme iron, and 15–35% of this form of iron is absorbed (Hurrell, 2002), whereas in plant foods, non-heme iron absorption is typically less than 10% (Zimmermann et al., 2005; Hurrell, 2002), as non-heme iron is heavily influenced by the presence of other food components (Hurrell and Egli, 2010).

Despite lower bioavailability, the quantity of dietary non-heme iron is largely greater than that of heme-iron in most diets (Abbaspour et al., 2014). In North America and Europe, two-third of the iron in the diet is mostly present as ferric form non-heme iron and one-third as heme iron (Carpenter and Mahoney, 1992). Therefore, even if the non-heme iron has lower bioavailability in diet, it generally contributes more than heme iron to iron nutrition. Non-heme iron is also the

primary form used in food fortification and supplements for purpose of combating the global prevailing IDA (Navas-Carretero et al., 2009).

Dietary factors may inhibit or enhance non-heme iron absorption. Factors such as calcium, phytate (Hallberg et al, 1989, Lynch, 2011; Hurrell, 2002) and certain type of phenolic compounds abundant in plants inhibit (Brune et al, 1989), whereas ascorbic acid, meat and fish enhance non-heme iron absorption (Teucher et al, 2004; Hurrell, 2002). It is believed that mineral status is determined by the bioavailability of the mineral rather than its absolute content in foods (Ganji and Kies, 1994, Griessen et al., 1999; Raul, 2000). Apart from a sufficient mineral intake, an effective absorption system is also important (Bronner, 1998; Lartey et al., 2000), and factors such as mineral solubility, paracellular permeability, pH and intestinal transit time all play a role in the regulation of mineral absorption (Bronner and Pansu, 1999).

In the current body of literature, there is some evidence suggesting that certain non-digestible oligosaccharides (also known as prebiotics), such as inulin and fructooligosaccharide (FOS), have positive effects on mineral absorption (Brommage et al., 1993; Delzenne et al., 1995), including iron (Ohta et al., 1995). As shown in Fig. 1.2, inulin is a linear chain of fructose capped by an end unit of glucose, and FOS is basically partial enzymatically hydrolyzed inulin (Roberfroid and Delzenne, 1998; Roberfroid et al., 1998). According to Yap et al. (2005), inulin showed a significant improvement in iron apparent absorption, apparent retention and net retention in infants who received 1.00 g/d inulin for 14 days.



A) Inulin (2≤n≤60)

B)Fructooligosaccharides (2≤n≤10)

Fig. 1.2 Structures of Inulin (A) and FOS (B) (Yeung et al., 2005 a)

To be considered prebiotics, short-chain carbohydrates should be non-digestible by intestinal and pancreatic enzymes, and be selectively utilized by probiotic bacteria in colon such as *Bifidobacteria* and *Lactobacilli*, with their growth and activity offering benefits to health (Cummings et al, 2001; Niba & Niba, 2003). Examples of these health benefits include enhanced mineral bioavailability (Yasuda et al., 2006; Ohta et al., 1995; van den Heuvel et al., 1999; Coudray et al., 2003), regulation of lipid metabolism (Delzenne et al., 1993; Levrat et al., 1994; Fiordaliso et al., 1995), and enhanced intestinal immunity (Rhodes, 1989;

Correa-Matos et al., 2003; Ten Bruggencate et al., 2003). While some minimally digestible complex carbohydrates such as β-glucans, dietary fibers, and resistant starches may also be considered prebiotics because of their potential fermentability by colonic bacteria (Cummings et al, 2001; Gibson and Roberfroid, 1995), inulin is the most common prebiotics found mainly in plants, fungi and bacteria (Yasuda et al., 2006). Inulin exists naturally in more than 36,000 plants and the most common sources are artichokes, asparagus, garlic, leeks, onions, banana, wheat and chicory roots (Gorski, 1997; Hendary and Wallace, 1993; Van Loo et al., 1995). In western countries, the average daily consumption of inulin has been estimated to be 3-11 g in Europe and 1-4 g in the United States (Van Loo et al., 1995; Moshfegh et al., 1999). Inulin and FOS are used increasingly in food product formulations and are considered important commercial prebiotics (Rao, 2001).

Inulin and FOS could not be digested in stomach and intestine as the fructosyl units were joined by β (2-1) glycosidic bonds which resist hydrolysis by human alimentary enzymes. In other words, they could reach the colon intact and be hydrolyzed and fermented by cecal and colonic *Bifidobacteria* and *Lactobacilli* with inulinase enzymes (Robinson, 1995; Roberfroid, 1993; Sarkar, 2007). Fermentation products, mainly short-chain fatty acids (SCFAs) and some other organic acids, would be produced and thus lower the pH environment of the cecum and colon (Gibson and Roberfroid, 1995), and may improve the solubility of minerals and trace elements, increase their concentrations in the liquid phase, and

increase their corresponding colonic absorption (Greger, 1999; Van den Heuvel et al., 1998).

Taken together, there are several possible mechanisms for inulin or FOS to exert an enhancing effect on iron absorption. First, inulin is fermented by colonic bacteria such as *Bifidobacteria* and *Lactobacilli* to produce organic acids (Sakuma, 2002). SCFAs such as acetic acid, propionic acid and butyric acid are the dominant products of fermentation (Topping and Clifton, 2001), and these acids decrease the pH of intestinal lumen, and may help to change iron from insoluble to soluble form, maintain iron solubility and thus increase diffusive absorption (Schulz et al., 1993; Younes et al., 1996). Interestingly, the concentrations of SCFAs are much higher in the right side of colon, including the cecal area, as it is the first location at which colonic bacteria ferment carbohydrate substrates, and hence the area has the highest fermentative activity for potential health benefits (Cummings, 1997). It is believed that many inulin-related health benefits are associated with an increase in SCFA production in the large intestine. Second, SCFAs may directly influence iron absorption by modifying various electrolytes exchanges (Trinidad et al., 1993). Third, according to Ohta et al., (1998a, 1998b), dietary FOS increased expression of intestinal calcium transport protein, calbindin-D9k, at the brush border in the cecum and colorectum in rats. Fermentation products of inulin could also have effects on iron transport proteins at the brush border of intestinal epithelial cells or other cellular proteins involved in the trafficking of iron across the mucosal barrier. The study by Tako et al., (2008) showed that dietary inulin led to a much higher

mRNA levels of DMT-1, Dcytb, ferroportin, ferritin and TfR in anemic piglets. In another pig study by Yasuda et al. (2006), 4% inulin supplementation to a corn/soybean diet after five weeks resulted in an increased Fe absorption and 15% higher hemoglobin concentration. Furthermore, enhanced proliferation and differentiation of different epithelial cells may also be associated with an enhancement in mineral bioavailability by inulin fermentation products (Yasuda et al., 2006).

1.4 Current issue and problem

The effect of prebiotics on mineral absorption have been studied both in animal and human for many years (Ohta et al. 1994; Ohta et al. 1995; Hubert et al. 2000; Yasuda et al. 2006; Younes et al. 2001, van den Heuvel et al. 1998). However, most of the studies were focused on calcium and magnesium. The studies on trace elements including iron were fewer and results were inconsistent and contradictory (Weber et al. 2010; Freitas et al. 2012; Petry et al. 2012). For example, Sakai et al. (2000b) reported that 7.5% inulin supplementation did not have any effects on recovery from post-gastrectomy anemia in rats, while Freitas et al. (2012) showed that rats fed 10% inulin or FOS in diets resulted in a significant higher hemoglobin concentration than the control, but 10% Synergy^{TM} (a 1:1 mixture of inulin and FOS) was not significant different from the control.

In addition, very few studies have been done in humans to investigate the potential benefits of FOS (Tahiri et al., 2003), and inconsistent results were obtained on iron absorption (Coudray et al. 1997; Van den Heuvel et al., 1998). Coudray et al. (1997) showed that inulin supplementation at 40 g/d did not increase iron absorption, but increased calcium absorption by about 50% in 9 healthy young men. In another study on 12 healthy young men using a stable-isotope technique, Van den Heuvel et al. (1998) showed that FOS at 15 g/d had no effects on either iron or calcium absorption. In both studies, the subjects were young men of adequate iron status. The effects of FOS supplementation on the iron status in iron deficient or anemic individuals remain unclear. Nevertheless, FOS

supplementation did not appear to negatively affect iron absorption in these two human studies. Although there are some inconsistencies between human studies and *in vitro*/animal studies, prebiotics such as inulin may still have an enhancing effect without showing any adverse effects on mineral absorption (van Loo et al., 1999).

Furthermore, whether inulin has to reach the colon for enhancement of iron absorption, or whether the colon is efficient as a site of iron absorption in response to inulin fermentation remains questionable. Results from the study by Patterson et al. (2009) in anemic pigs suggested that colon is not an important site for iron absorption and inulin did not influence colonic iron absorption. However, a trial with iron-deficient dogs showed that colon can be a significant site for iron absorption for iron deficient subjects (Yeung et al., 2005a). Besides, it is also unclear that which form of inulin (unfermented or fermented) will affect the expression of iron regulatory genes and what the effects of inulin on anemic individuals would be when compared to iron-adequate ones, and whether any enhancing effects are dependent on the bioavailability of the iron in the meal. Further investigations are imperative for a much better understanding of the potential enhancing effects of inulin on iron absorption.

1.5 Research Objectives

There were two main experimental objectives in this research project. The first main objective was to determine the effects of dietary prebiotics, FOS and some other prebiotics, on iron status in iron-adequate and iron-deficient rats.

The second main objective of this project was to expose human intestinal epithelial cells to FOS and its fermentation products, the SCFAs, and to quantify the kinetics of the expression of iron regulatory genes as a function of exposure time.

The long-term goals of this research were to analyze and establish whether FOS or other non-digestible oligosaccharides would enhance iron absorption as bioactive carbohydrates and dietary prebiotics, and to elucidate the mechanisms involved.

1.6 Significance

This research project is significant and relevant because ID is the most prevalent nutrient deficiency in the world. Poor bioavailability of dietary iron, especially from diets high in cereals and legumes, is a major factor contributing to ID. One effective, but often neglected, strategy for alleviating ID is dietary modification to increase intake of dietary components that promote iron absorption from low-bioavailability meals. Dietary prebiotics such as inulin and FOS which are naturally present in a wide variety of plant foods may enhance iron absorption from diets with low iron bioavailability, but very few studies have been reported and mechanisms involved are not well understood. Furthermore, there is currently a lack of studies in the literature that compare multiple prebiotics in a single experiment.

Increasing the amount of iron in the body through increased consumption of iron-enriched or fortified foods, or through the use of supplements have not always been successful, especially in developing countries when compliance issues are common (Abrams, 1999). So it is reasonable to expect that an increase in the absorption efficiency of iron from normal daily diets would not only make it easier for individuals at risk to fulfil their daily requirements, but would also minimize compliance issues. Therefore, a more thorough understanding of the role of FOS in iron absorption and the mechanisms involved may lead to new strategies for preventing iron deficiency and provide another reason for promoting plant-based diets rich in FOS or other prebiotics.

Chapter 2 Effect of FOS and other prebiotics on iron status in SD rat model

2.1 Introduction

According to FAO (2007), a prebiotic is "a non-viable food component that confers a health benefit on the host associated with modulation of the microbiota". A newer concept had also been introduced by Gibson et al. in 2010, which suggested that "a dietary prebiotic is a selectively fermented ingredient that results in specific changes, in the composition and/ or activity of the gastrointestinal microbiota, thus conferring the benefit(s) upon host health." Prebiotics, generally speaking, are non-digestible oligosaccharides that selectively stimulate the growth and activities of specific species of bacteria in the colon, usually *Bifidobacteria* and *Lactobacilli*, with benefits to health (Cummings, Macfarlane and Englyst, 2001; Roberfroid, 2000).

Only few compounds of carbohydrates have been regarded as prebiotics, most noticeably, short- and long- chain β-fructans such as fructooligosaccharide (FOS) and inulin, galactooligosaccharide (GOS) as well as lactulose (Valcheva and Dieleman, 2016). FOS and inulin are fructans because they both contain GpyF*n* (α-D-glucopyranosyl-[β-D-fructofuranosyl]$_{n-1}$-D-Fructofuranoside) and FpyF*n* (β-D-fructopyranosyl-[β-D-fructofuranosyl]$_{n-1}$-D-Fructofuranoside). The number of fructose units could range from 2 to more than 70, and fructans contain less than or equal to 10 units are typically considered FOS (Gibson et al., 2010). While

inulin is naturally found in hundreds of different plant foods (van Loo et al., 1995), FOS is usually obtained through hydrolysis of inulin by inulinase (Crittenden, 1999). Both inulin and FOS (or their mixtures) are commercially available as ingredients for foods and beverages (Gorski 1997). For example, Synergy^{TM} is a commercial product blending 50% short-chain FOS with 50% long-chain inulin (Gibson et al., 2010).

GOS, naturally present at a low level in breast milk, is a galactose-containing oligosaccharide with a terminal glucose unit. Commercially produced GOS is derived from lactose by transglycosylation action of β-galactosidases. The degree of polymerization (DP) of GOS is usually 2-5 in the form of Glu α1-4[Gal β1-6]$_n$ (Crittenden, 1999; Whisner and Weaver, 2013). Lactulose is a synthetic disaccharide, also manufactured from lactose like GOS, but through an isomerization process in which the glucose moiety in the lactose molecule is converted to fructose, resulting in the final form of Gal β1-4 Fru (Gibson et al., 2010). Although non-digestible oligosaccharides generally contain at least three monosaccharide units, lactulose has similar prebiotic properties and therefore, is also commonly included in the prebiotics class (Crittenden and Playne, 1996).

All these prebiotics had been reported to stimulate the growth and proliferation of probiotic bacteria in the colon with various health-promoting effects. Langlands et al. (2004) showed that FOS supplementation of diets increased surface counts of *Bifidobacteria* and *Lactobacilli* in biopsy samples taken from the cecum, transverse and descending colon, and rectum of human subjects

during colonoscopy. Ito et al. (1990) reported a significant increase in *Bifidobacteria* and *Lactobacilli* at different test doses (2.5, 5, 10 g/day) in 12 men. Gibson et al. (1995) also showed that 15 g/day intake of FOS and inulin could stimulate *Bifidobacteria* growth after two weeks and the effect lasted as long as prebiotics were consumed. Lactulose has also been shown to increase *Bifidobacteria* and *Lactobacilli* both *in vitro* and *in vivo* (Fadden and Owen, 1992; Suzuki et al., 1985; Terada et al., 1992).

Proliferation of beneficial bacteria in the colon has been linked to a number of health benefits, including enhanced defense against pathogens, reduction of toxic metabolites and detrimental enzymes, and prevention of constipation (Spiegel et al., 1994), modulation of the immune system (Jeurink et al, 2013), and regulation of neuroendocrine stress response (Schmidt et al., 2015). Furthermore, prebiotics could potentially influence mineral absorption as studies have shown that prebiotics supplementation exerts positive effects on absorption and availability of calcium (Ohta et al. 1995; Ohta et al. 1998; Weaver et al. 2011), magnesium (Ohta et al. 1994; Ohta et al. 1995; Delzenne et al. 1995), iron (Asvarujanon et al. 2005; Wang et al. 2010), zinc (Coudray et al. 2006) and copper (Coudray et al. 2006). However, data on the effect of prebiotics on iron absorption from *in vivo* studies are inconsistent. Yeung et al. (2005a) suggested that prebiotics may have an enhancing effect on iron absorption, and several biologically plausible mechanisms have been proposed. While a stable isotope study by Patterson et al. (2009) with anemic piglets surgically fitted with cecal cannulas showed that FOS had no enhancing

effect on iron absorption in the colon, Tako et al. (2008) observed that FOS supplementation triggered an upregulation of genes encoding iron transporters in the enterocytes from the duodenum and colon of anemic piglets. In another study using the porcine model, Samolińska and Grela (2016) reported increases in iron, zinc and copper concentrations in blood plasma during the fattening period with FOS supplementation.

In a randomized controlled intervention trial among young children in India, Sazawal et al. (2010) showed that children aged 1-3 receiving milk fortified with a combination of *B. lactis* HN109 (1.9 x 107 97 CFU/d) and "prebiotic oligosaccharide" (2.4 g/d) for 1 year resulted in a 34% reduction in iron-deficiency anemia and a weight gain of 0.13 kg/year, when compared to a control group receiving the same milk without fortification. Nevertheless, hemoglobin and hematocrit levels were not different between the two groups. It was undefined whether the reduction in anemia was due to enhanced iron absorption, or due to an overall improvement of the health status of the children. Data from other human studies focusing on the direct effects of prebiotics on enhancing iron absorption are quite limited. Coudray et al. (1997) showed that inulin supplementation at 40 g/d did not increase iron absorption, but increased calcium absorption by about 50% in 9 healthy young men. In another study on 12 healthy young men using a stable-isotope technique, Van den Heuvel et al. (1998) showed that FOS at 15 g/d had no effects on either iron or calcium absorption. In both studies, the subjects were young men of adequate iron status. The effects of FOS supplementation on the

iron status in iron deficient or anemic individuals remain unclear. Similarly, conflicting data have also been reported in studies involving inulin, GOS and lactulose. It had been shown that inulin did not influence fractional iron absorption in women with low iron status (Petry et al. 2012), and 7.5% inulin supplementation did not have effect on recovery from post-gastrectomy anemia in rats (Sakai et al., 2000b). Freitas et al. (2012) showed that rats fed 10% inulin or FOS in diets resulted in a significant higher hemoglobin concentration than the control, but 10% Synergy^{TM} was not significant different from the control. Van den Heuvel et al. (1998) demonstrated that 15 g of GOS, inulin or FOS supplementation to 12 healthy men for three weeks had no effect on iron and calcium absorption. Nagendra et al. (1994) reported that there were no significant differences in absorption and retention of nitrogen, calcium, phosphorus and iron in rats fed with infant formula with or without 0.5 – 1.0 % lactulose.

The primary strategies for compacting iron deficiency are dietary modification, iron supplementation and fortification of foods, and biofortification through plant-breeding to improve iron intake and bioavailability (Zimmermman and Hurrell, 2007). When meat and fish are low in diets and food iron has low bioavailability, iron absorbed from foods simply would not be enough to prevent iron deficiency in many women and children, especially in developing countries, even though iron absorption efficiency increases when iron storage is depleted (Zimmermman and Hurrell, 2007; Benito et al. 1997). As a result, new strategies and knowledge on dietary composition must be emphasized.

The effect of prebiotics on mineral absorption have been studied both in animal and human for many years (Ohta et al. 1994; Ohta et al. 1995; Hubert et al. 2000; Yasuda et al. 2006; Younes et al. 2001, van den Heuvel et al. 1998). However, most of the studies were focused on calcium and magnesium. The studies on trace elements including iron were fewer and results were inconsistent and contradictory (Weber et al. 2010; Freitas et al. 2012; Petry et al. 2012). As to the studies on iron absorption or bioavailability, most research was carried out with fructans including FOS, inulin and Synergy^{TM}, and to a lesser extent, GOS and lactulose. There is a lack of studies that compare the effects of all these different prebiotics together in a single experiment.

The focus of this part of research project was to determine the effects of prebiotics on iron status. In the first experiment, the objectives were to determine the effect of FOS supplementation on the iron status of anemic and normal male Sprague-Dawley rats, and to determine if FOS supplementation would promote the recovery of anemic rats. In the second experiment, the objective was to determine and compare the effects of different type of prebiotics including FOS, inulin, Synergy^{TM}, GOS and lactulose on the iron status of anemic rats.

2.2 Materials

2.2.1 Chemicals

Chemicals and reagents used in tissue non-heme iron analysis and short chain fatty acid analysis were obtained from Sigma-Aldrich Inc. China (Shanghai, China) or Fisher Scientific (Guangzhou, China) unless stated otherwise: Hydrochloric acid (HCl), Trichloroacetic acid (TCA), sodium 3-(2-pyridyl)-5,6,-bis(4-phenylsulfonate)-1,2,4-triazine (ferrozine), sodium acetate, thioglycolic acid, iron standard (TraceCERT®, 1000 mg/L in nitric acid), 2-propanol, pyridine, propyl chloroformate (PCF), hexane, sodium sulfate anhydrous and DL-2-Methylbutyric acid were purchased from TCI (Shanghai, China). FOS (BENEO Orafti® P95 Oligofructose, powder), inulin (BENEO Orafti® GR, powder, DP ≥10 and BENEO Orafti® Synergy1, oligofructose-enriched inulin, powder) were obtained from the Food Ingredients Division of Guangzhou DPO Co. Ltd (Guangzhou, China). GOS (King-Prebiotics, powder) was obtained from New Francisco (Yunfu City) Biotechnology Co. Ltd (China). Lactulose (Duphalac®) was obtained from K.L.Medicine (Hong Kong). Water used in experiments was purified with the Milli-Q Reference Ultrapure water purification system (EMD Millipore, Billerica, MA).

2.2.2 Animals

Weanling two-week-old male Sprague-Dawley (SD) rats with a mean body weight of <40g were purchased from Laboratory Animal Unit of The Chinese

University of Hong Kong (Shatin, Hong Kong SAR). Upon arrival, they were housed in a temperature-controlled room in plastic cages with stainless-steel cover, on a 12-hour dark-light cycle.

2.2.3 Diets

Test diets were based on a commercial purified AIN-93G rodent diet, containing either 12 μg Fe/g diet (low-iron diet) or 45 μg Fe/g diet (regular diet). All diets used in this study were prepared by Trophic Animal Feed High-tech Co. Ltd (Nantong, China).

2.3 Methods

2.3.1 Experimental design

Two animal studies had been conducted. Both experimental protocols were approved by The Government of The Hong Kong Special Administrative Region, Department of Health (License No. (15-37) in DH/HA&P/8/2/6 Pt.4). All rat experiments were conducted in animal facilities at Hong Kong Baptist University (HKBU).

The experimental design was similar in both studies, with the main difference being the grouping of prebiotic treatments. In Experiment 1, a total of 48 rats were used in the experimental design (Table 2.1). Upon arrival, rats were divided into 8 groups of 6 rats according to the average body weight (equal mean weights across groups). During the acclimation period, 5 groups received the low-iron diet while the other 3 groups received the regular diet for 14 days. All rats had free access to the diet and water. On Day 14, 1 group from each diet treatment was sacrificed to obtain baseline data on hemoglobin concentration, as well as liver, spleen, kidney and heart non-heme iron levels to confirm iron-deficiency anemia.

Table 2.1 Animal Experiment 1 design

Experiment period	**Day 1 – 14 (Acclimation)**	**Day 15 – 42 (Feeding trial)**	
	Fe level (μg/g diet)	Fe level (μg/g diet)	FOS level (w/v in water)
Diet treatments and levels	45 (Normal)	45	0%
		45	5%
	12 (Anemic)	45	0%
		45	5%
		12	0%
		12	5%

Note: In addition to the 6 treatment groups (n=6) shown in the experimental design, 1 Normal group and 1 Anemic group were included for tissue non-heme iron analysis after the acclimation period (Day 14) to confirm iron-deficiency. So, 8 groups of 6 rats for a total of 48 rats were used in Experiment 1.

At the onset of the feeding trial (Day 15), 2 "Anemic" groups were kept on the same low-iron diet with or without FOS supplementation, while the other 2 "Anemic" groups were switched to the regular diet, also with or without FOS supplementation to allow recovery from anemia. FOS was provided to the rats by completely dissolving in water at 5% w/v. Rats were individually caged and had free access to the diet and water. Our preliminary trial revealed that rats under these conditions on average consumed roughly the same amount of water and gained weight at about the same rate with or without FOS (dissolved in water at 5% w/v). The 2 "Normal" groups were kept on the same regular diet, also with or

without FOS supplementation. All rats would receive their respective test diets for 28 more days (Day 15 - 42).

In Experiment 2, a total of 80 weaning SD rats were distributed to 10 groups (n=8) according to Table 2.2. Similar to Experiment 1, rats would go through the acclimation period and 1 group from each diet treatment was sacrificed to obtain baseline data to confirm iron-deficiency anemia.

Table 2.2 Animal Experiment 2 design

Experiment period	**Day 1 – 14 (Acclimation)**	**Day 15 – 49 (Feeding trial)**	
	Fe level (μg/g diet)	Fe level (μg/g diet)	Prebiotics level (w/v in water)
Diet treatments and levels	45 (Normal)	45	0%
		45	5% Synergy1
	12 (Anemic)	12	0%
		12	5% Synergy1
		12	5% FOS
		12	5% Inulin
		12	5% GOS
		12	5% Lactulose

Note: In addition to the 8 treatment groups (n=8) shown in the experimental design, 1 Normal group and 1 Anemic group were included for tissue non-heme iron analysis after the acclimation period (Day 14) to confirm iron-deficiency. So, 10 groups of 8 rats for a total of 80 rats were used in Experiment 2.

At the onset of the feeding trial (Day 15), the 2 “Normal” groups were kept on the same regular diet supplemented with or without 5% Synergy1, the other 6

"Anemic" groups were kept on the same low iron diet with or without supplementation of different prebiotics according to Table 2.2. All prebiotics were provided in the same way as FOS in Experiment 1 by dissolving in water at 5% w/v. Rats had free access to their respective test diets and water for 35 more days (Day 15 - 49).

In both experiments, rats were observed daily during the whole study for signs of abnormalities. The body weight of each rat was measured every week, and water consumption was recorded every 1-2 days. Blood samples were drawn weekly for hemoglobin concentration measurements. At the end of the feeding trials, all rats were sacrificed. Liver, spleen, kidney and heart samples were harvested, and weighed portions were used for non-heme iron analysis.

2.3.2 Hemoglobin concentration measurement

Whole blood samples were collected from the tail vein of rats each week during the feeding trial period. A commercial hemoglobin assay kit (Pointe Scientific, Inc., Canton, MI) was used and the manufacturer's protocol was followed to measure the hemoglobin concentration of the blood samples.

Briefly, 2.0 mL of commercial hemoglobin reagent and 10 μL of standard or samples were first mixed in a test tube, and stood for 3 min at room temperature. Absorbance at 540 nm of the samples and the standard (11.5 g/dL) was measured by UV-VIS spectrophotometer (Shimadzu UV-1700, Kyoto, Japan). Hemoglobin

concentration was calculated using the following formula: Hemoglobin (g/dL) = (Abs. of sample/ Abs. of standard) x concentration of standard (g/dL).

2.3.3 Tissue non-heme iron analysis

Rats were first anaesthetized through exposure to diethyl ether. Liver, spleen, kidney and heart were harvested after laparotomy, rinsed with a saline solution (9 g/L), and stored at -80 ^{o}C until analysis. Tissue non-heme iron levels were determined by the colorimetric method described by Rebouche et al. (2004). Briefly, around 1 mL of tissue homogenates were prepared in Milli-Q (1:10 w/v) by using a glass homogenizer. Then 500 μL of tissue homogenate from each sample was transferred to a 1.5 mL centrifuge tube and mixed with equal volume of protein precipitation solution (1N HCl and 10% TCA in Milli-Q), followed by heating for 1 hr at 95 ^{o}C. Afterwards, tubes were cooled in water for 5 min, vortexed and centrifuged at 12,000 rpm for 10 min. Supernatant of each sample was carefully divided into 2 aliquots of 300 μL (each in a new 1.5 mL centrifuge tube). One tube was mixed with the chromogen solution (0.508 mmol/L ferrozine, 1.5 mol/L sodium acetate and 1.5% v/v thioglycolic acid in Milli-Q) for iron chromogenic reaction. The other tube served as the blank and mixed with the sample blank solution (1.5 mol/L sodium acetate and 1.5% v/v thioglycolic acid in Milli-Q). After 30 min at room temperature, absorbance was measured at 562 nm by UV-VIS spectrophotometer (Shimadzu UV-1700, Kyoto, Japan).

Standard curves were prepared freshly from iron standard solutions at 0, 2, 4, 6, 8, 10 μg/mL on the day of tissue analysis. Results were expressed as μg Fe/g tissue (wet weight).

2.3.4 Short chain fatty acid (SCFA) analysis by GC-MS

At the end of Experiment 2, rats were sacrificed and rat colon samples including the colon contents were collected, rinsed with a saline solution (9 g/L), and stored at -80 ^{o}C until analysis.

a. **Standard solution preparation**: Accurately weighed standards of acetic acid, propionic acid, butyric acid, isobutyric acid, pentanoic acid and isopentanoic acid were used to prepare the standard solutions at 7 concentrations: 1 μg/mL, 10 μg/ mL, 20 μg/ mL, 50 μg/ mL, 100 μg/ml, 250 μg/ mL and 500 μg/ mL.

b. Standard pretreatment: One mL of standards was added to 50 μL of DL-2-methylbutyric acid (the internal standard), vortex mixed for 2 min and left standing for 2 hr. It was then vortexed for 2 more min and centrifuged at 13,000 rpm at 4 ^{o}C for 20 min. After centrifuge, 500 μL of supernatant was transferred to a centrifuge tube, and 300 μL of Milli-Q, 500 μL of isopanol/pyridine solution (3:2 v/v) and 100 μL of PCF were added and gently vortexed for 30s, followed by ultrasonication for 1 min at room temperature for derivatization reaction. Afterwards, a two-step extraction was performed. First, 300 μL of hexane was added to the mixture and vortex mixed for 1 min, and then centrifuged at 3000 rpm for 5 min. The upper hexane layer was transferred to a new centrifuge tube.

Second, the extraction was repeated by adding another 200 μL of hexane to the mixture residue. The upper hexane layers from the two extractions were combined and mixed with sodium sulfate anhydrous to remove trace of water, vortexed for 30s and centrifuged at 3000 rpm for 5 min. The supernatant was drawn for GC-MS analysis.

c. Sample pretreatment: All sample pretreatment steps were performed at 4 ^{o}C to preserve the volatile SCFAs. The colon samples were first thawed on ice, and colon content samples were extruded, weighed and recorded accurately. The samples were put into glass centrifuge tubes, and 1 mL of 0.005 M NaOH solution and 50 μL of DL-2-methylbutyric acid were added to the tubes. Subsequent steps were the same as for standard pretreatment.

d. GC-MS analysis conditions: Agilent HP-5 capillary column (30 m x 0.35 mm ID, 0.25 μm thickness) with an injection volume of 1 μL was used. The initial temperature was held at 60 ^{o}C for 5 min, then ramped up to 250 ^{o}C at 10 ^{o}C /min. Helium was used as carrier gas at the flow rate of 1.0 mL/min. The MS detector utilized electron impact (EI) ion source and collected data by SIM scan mode. Temperature settings (front inlet: 280 ^{o}C, ion source: 230 ^{o}C, transfer line: 250 ^{o}C) were used and the electron energy was -70 eV.

2.3.5 Statistical analysis

All statistical analyses were done using IBM SPSS Statistics (Statistical Product and Service Solutions, version 21.0). Differences in body weight, water

consumption, hemoglobin concentration and tissue non-heme iron in rats with or without prebiotics were analyzed by t-test. In Experiment 2, effects of different prebiotics in anemic rats were analyzed by one-way ANOVA and Post Hoc comparisons. Values were given as Mean ± SEM. A p-value of <0.05 was considered significant.

2.4 Results

2.4.1 Animal Experiment 1

2.4.1.1 Acclimation and iron-deficiency anemia

After two weeks of acclimation, rats received regular iron diet showed a normal iron status containing about 12 g/dL hemoglobin, while rats in low-iron diet groups became anemic with a hemoglobin concentration at about 5 g/dL. The anemic rats not only showed a lower body weight, but also lower non-heme tissue iron levels in liver, spleen, kidney and heart than normal rats (Table 2.3 and Fig. 2.1). These differences indicated a slower growth and a confirmation of iron deficiency anemia.

2.4.1.2 Weight gain and water intake

During the whole experiment, the weight of all rats increased linearly (Fig. 2.2). Daily observations showed that there were no signs of diarrhea in rats. There were no significant differences in body weight between each pair of rat groups with or without FOS at any time points (i.e. Normal vs. Normal w/FOS; Anemic – Regular vs. Anemic – Regular w/FOS; Anemic – Low-iron vs. Anemic – Low-iron w/FOS on Day 14, Day 21, Day28, Day 35 or Day 42) (Table 2.4).

Table 2.3 Body weight, hemoglobin concentration and tissue non-heme iron levels (Mean ± SEM) of normal and anemic rats after the acclimation period (Day 14) in Experiment 1

	Normal	**Anemic**
Body weight (g)	101.9 ± 4.5[a]	87.0 ± 3.2[b]
Hemoglobin (g/dL)	12.3 ± 0.3[a]	5.0 ± 0.3[b]
Liver non-heme iron (µg/g)	50.0 ± 2.6[a]	13.7 ± 0.8[b]
Spleen non-heme iron (µg/g)	45.9 ± 6.1[a]	15.9 ± 0.9[b]
Kidney non-heme iron (µg/g)	17.7 ± 0.9[a]	8.6 ± 0.5[b]
Heart non-heme iron (µg/g)	21.7 ± 0.9[a]	11.3 ± 0.8[b]

[a,b] Means with different superscripts within the same row are significantly different ($p<0.05$).

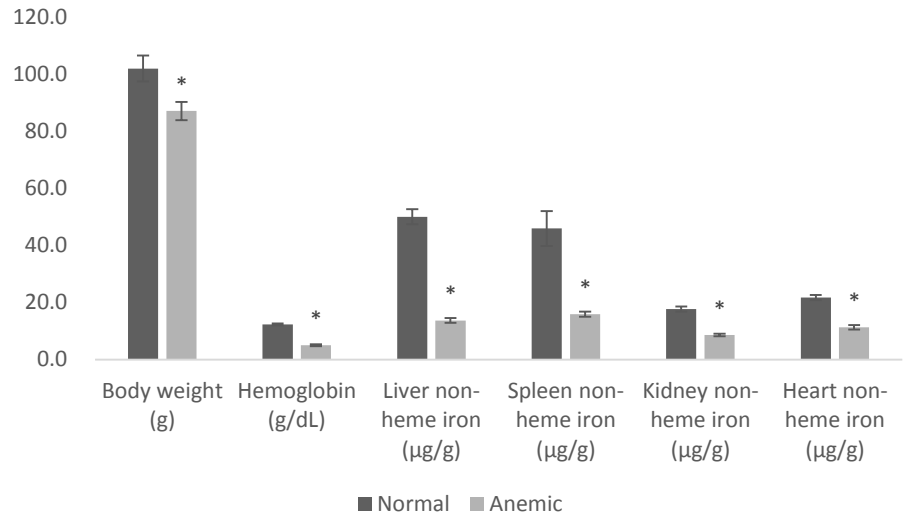


Fig. 2.1 Body weight, hemoglobin concentration and tissue non-heme iron levels (Mean ± SEM) of normal and anemic rats in Experiment 1 on Day 14.
*Significant difference ($p<0.05$) between normal and anemic rats

Anemic rats with or without FOS gained weight slower than normal rats. But the anemic rats switched to the regular diet showed the same growth rate as normal rats with or without FOS (Fig. 2.2).

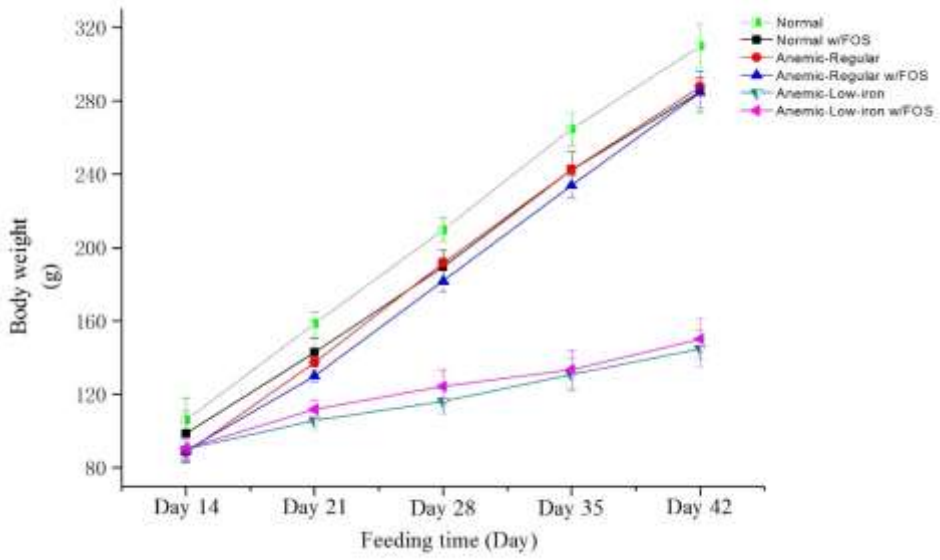


Fig. 2.2 Weekly changes in body weight (g, mean ± SEM) of rats in Experiment 1

Table 2.4 Body weight of rats (g, mean ± SEM) in Experiment 1

Treatment groups	Day 14	Day 21	Day 28	Day 35	Day 42
Normal	106.1 ± 12.1	158.6 ± 6.2	209.9 ± 6.9	264.6 ± 9.2	310.0 ± 12.0
Normal w/FOS	98.9 ± 11.9	143.0 ± 7.4	189.6 ± 9.0	242.3 ± 10.0	285.1 ± 11.0
Anemic - Regular	88.3 ± 5.2	137.5 ± 2.6	191.8 ± 2.5	242.4 ± 3.0	287.8 ± 4.4
Anemic - Regular w/FOS	89.5 ± 6.2	130.1 ± 3.6	181.9 ± 6.0	233.9 ± 6.7	284.8 ± 8.0
Anemic - Low-iron	90.4 ± 6.3	105.7 ± 4.6	116.3 ± 6.9	130.9 ± 8.5	144.9 ± 10.0
Anemic - Low-iron w/FOS	90.6 ± 5.9	112.0 ± 5.3	124.5 ± 8.7	133.7 ± 10.0	150.3 ± 11.0

There were also no significant differences in daily water intake during the feeding trial between paired groups of rats with or without FOS (Table 2.5). The only exception was observed in the pair of Anemic –Regular vs. Anemic – Regular w/FOS groups during the first week (Day 15-21). The rats fed with FOS consumed less water than rats in the same diet without FOS.

Overall, FOS did not affect the weight gain in normal or anemic rats, and the feeding of 5% w/v FOS by water did not alter the pattern of water consumption for rats in this experiment.

Table 2.5 Daily water intake (mL, mean ± SEM) during the feeding trial in Experiment 1

Treatment groups	Day 15-21	Day 22-28	Day 29-35	Day 36-42	Total Mean
Normal	26.6 ± 1.4	40.1 ± 2.0	41.8 ± 2.1	44.0 ± 1.5	38.1 ± 3.7
Normal w/FOS	26.2 ± 1.7	38.4 ± 2.1	43.2 ± 3.3	46.5 ± 2.7	38.6 ± 5.2
Anemic - Regular	20.4 ± 0.7	25.2 ± 0.7	21.3 ± 1.0	22.6 ± 1.4	22.4 ± 2.2
Anemic - Regular w/FOS	17.6 ± 0.8*	23.8 ± 1.3	22.2 ± 1.0	26.3 ± 1.2	22.5 ± 1.4
Anemic - Low-iron	13.3 ± 0.6	18.0 ± 0.8	18.2 ± 1.1	20.7 ± 1.2	17.5 ± 2.0
Anemic - Low-iron w/FOS	13.4 ± 0.6	18.2 ± 0.8	19.4 ± 1.5	21.1 ± 1.7	18.0 ± 2.5

* Significant difference between Anemic-Regular rats with and without FOS ($p<0.05$).

2.4.1.3 Hemoglobin concentration and non-heme iron level during the feeding trial

At the beginning of the feeding trial (Day 14), hemoglobin concentration of anemic rat groups was within a range of 5.0 – 5.6 g/dL (Table 2.6). The groups of anemic rats kept on the same low iron diet continued to show low hemoglobin concentration throughout the feeding trail. However, there was a mild increase ($p<0.05$) in hemoglobin concentration for anemic rats remaining the low-iron diet with FOS supplementation compared to those without FOS after 3 weeks (Day 35) (Table 2.6 and Fig. 2.3). The Anemic – Low-iron rats supplemented with FOS maintained a higher hemoglobin concentration than rats without FOS after 4 weeks (Table 2.6), and the difference was close to statistical significance ($p=0.088$). In addition, when the rats were sacrificed at the end of the trail, the Anemic – Low-iron rats with FOS supplementation also showed a significant higher liver non-heme iron level (Table 2.7 and Fig 2.4a), while there were no differences in non-heme iron levels in spleen, kidney and heart.

Table 2 6 Hemoglobin concentration of rats (g/dL, mean ± SEM) in Experiment 1

Treatment groups	Day 14	Day 21	Day 28	Day 35	Day 42
Normal	13.4 ± 0.3	12.1 ± 0.4	11.8 ± 0.3	15.6 ± 0.4	14.1 ± 0.3
Normal w/FOS	12.8 ± 0.4	12.9 ± 0.3	12.9 ± 0.5	15.5 ± 0.3	14.8 ± 0.4
Anemic - Regular	5.0 ± 0.2	10.5 ± 0.2	12.7 ± 0.4	15.4 ± 0.1	14.6 ± 0.1
Anemic - Regular w/FOS	5.4 ± 0.2	10.8 ± 0.2	13.3 ± 0.4	14.5 ± 0.2	14.4 ± 0.2
Anemic - Low-iron	5.2 ± 0.2	4.6 ± 0.1	4.2 ± 0.3	4.5 ± 0.2	4.6 ± 0.1
Anemic - Low-iron w/FOS	5.6 ± 0.3	4.8 ± 0.2	4.6 ± 0.3	5.8 ± 0.4*	5.4 ± 0.3

* Significant difference between Anemic-Low-iron rats with and without FOS ($p<0.05$).

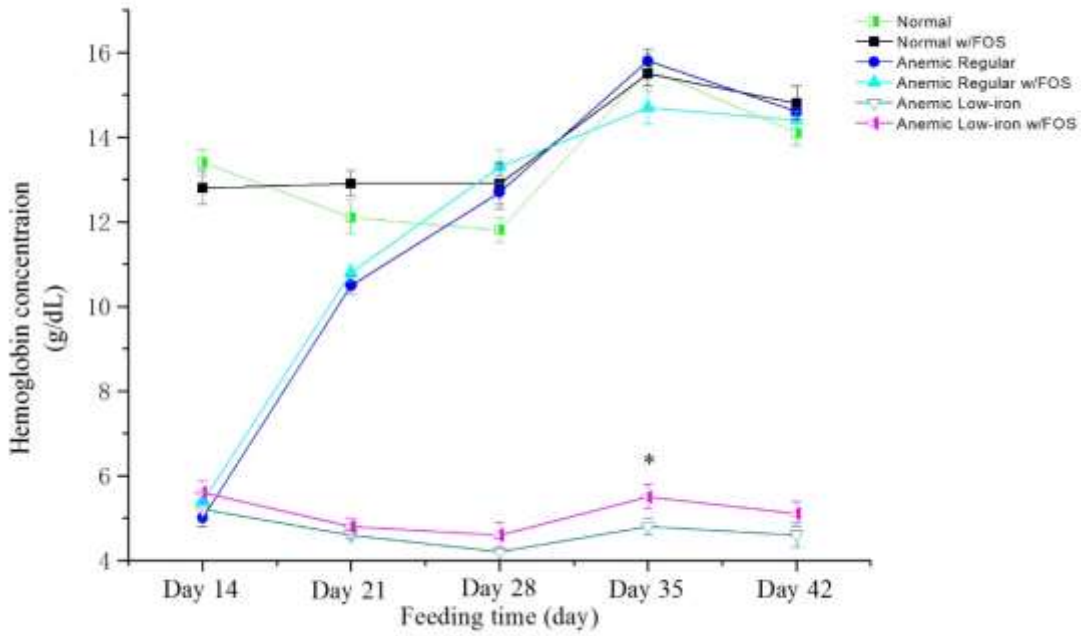


Fig. 2.3 Weekly changes in hemoglobin concentration (g/dL, mean ± SEM) of rats in Experiment 1. *Significant difference between Anemic-Low-iron rats with and without FOS ($p<0.05$).

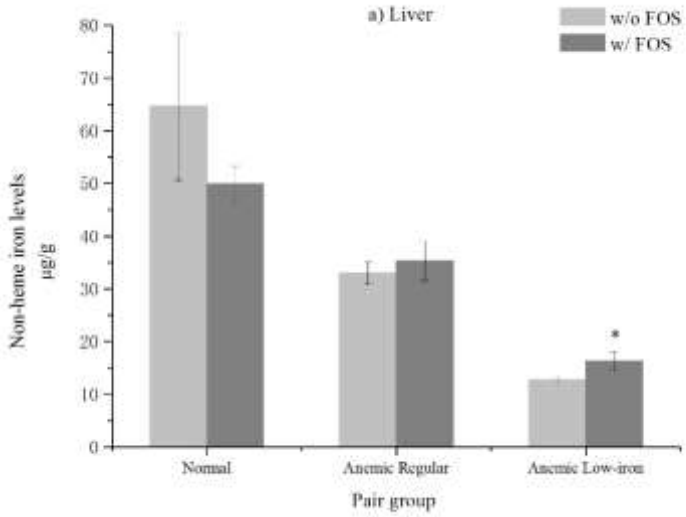
As to the pair groups of Anemic – Regular and Anemic – Regular w/FOS, hemoglobin concentrations of anemic rats switched to regular diet with or without FOS on Day 14 rose to about 10.0 g/dL after 1 week and were comparable to normal rats after only 2 weeks (Table 2.6 and Fig 2.3). During these 2 weeks of recovery, FOS supplementation did not provide any additional assistance in enhancing iron status as there were no significant differences between the rats with or without FOS on Day 21 or Day 28. Besides, the tissue non-heme iron levels in liver, spleen, kidney and heart showed no difference as well at the end of the trial (Table 2.7 and Fig 2.4).

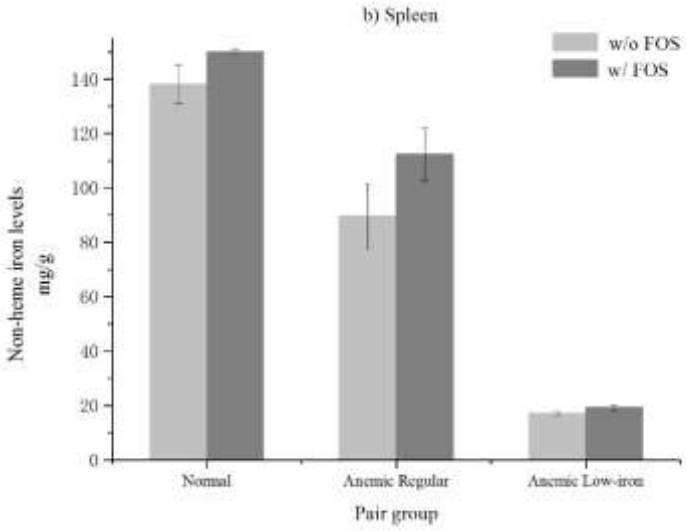
For normal rats supplemented with or without FOS, there were no significant differences in hemoglobin concentration at any time points or tissue non-heme iron levels at the end.

Table 2.7 Rat non-heme iron levels (μg/g, Mean ± SEM) after the feeding trial (Day 42) in different tissues in Experiment 1

Treatment groups	Liver	Spleen	Kidney	Heart
Normal	64.6 ± 14.0	138.2 ± 7.0	21.1 ± 1.1	26.7 ± 1.2
Normal w/FOS	49.9 ± 3.3	150.0 ± 2.0	20.0 ± 0.6	27.7 ± 1.1
Anemic - Regular	33.0 ± 2.1	89.7 ± 12.0	20.9 ± 1.4	25.6 ± 1.5
Anemic - Regular w/FOS	35.3 ± 3.7	112.4 ± 9.8	19.7 ± 0.7	26.5 ± 2.4
Anemic - Low-iron	12.8 ± 0.6	17.2 ± 0.9	10.6 ± 0.8	19.5 ± 2.6
Anemic - Low-iron w/FOS	14.7 ± 0.5*	19.2 ± 0.9	9.4 ± 0.4	18.0 ± 0.9

*Significant difference between Anemic-Low-iron rats with and without FOS ($p<0.05$).


a) Liver
w/o FOS
w/ FOS
Non-heme iron levels
μg/g
80
70
60
50
40
30
20
10
0
*
Normal
Anemic Regular
Anemic Low-iron
Pair group


b) Spleen
w/o FOS
w/ FOS
Non-heme iron levels
mg/g
140
120
100
80
60
40
20
0
Normal
Anemic Regular
Anemic Low-iron
Pair group

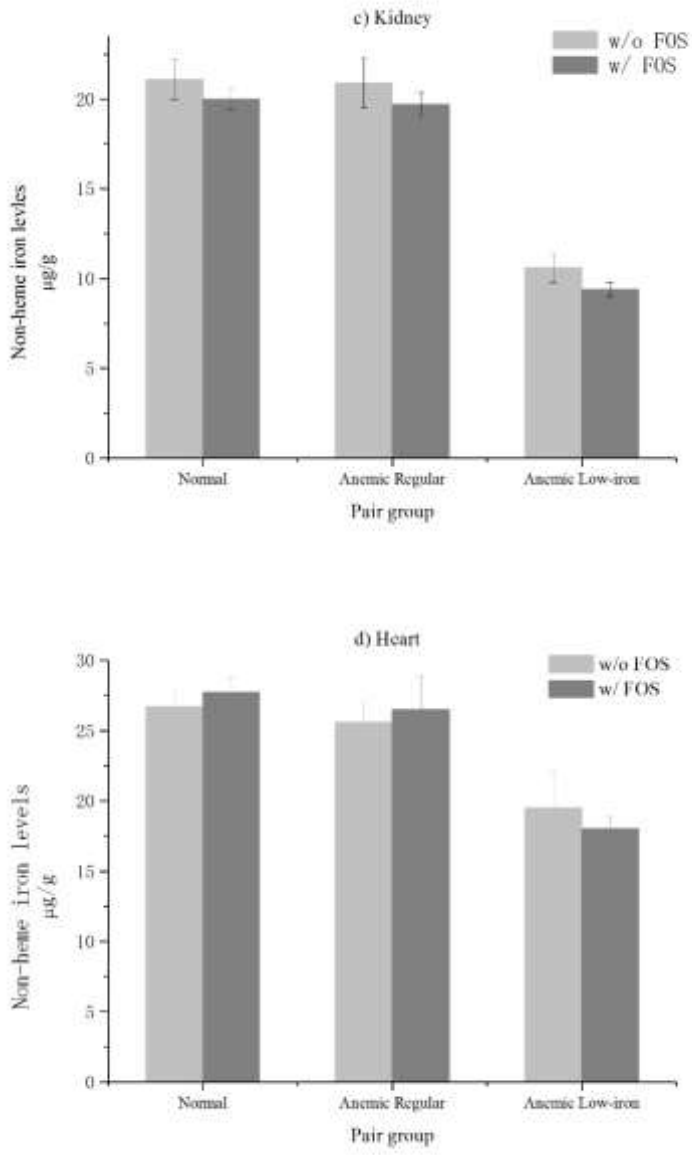


Fig. 2.4 Rat tissue non-heme iron levels (μg/g) after the feeding trial (Day 42) in a) liver; b) spleen; c) kidney; d) heart in Experiment 1. *Significant difference in liver non-heme iron levels between Anemic-Low-iron rats with and without FOS ($p<0.05$).

The results showed that the FOS supplementation could mildly increase the hemoglobin concentration and liver non-heme iron level in anemic rats with a low iron intake, but did not affect normal rats or provide any additional effect on the

recovery of anemic rats when the diet was switched from low-iron to regular in this experiment.

2.4.2 Animal Experiment 2

2.4.2.1 Acclimation and iron-deficiency anemia

Results were consistent with those in Experiment 1. After 14 days of acclimation, rats received the regular diet showed a normal iron status with a hemoglobin concentration of 14 g/dL, and those received the low-iron diet achieved iron-deficiency anemia with a hemoglobin concentration of 5 g/dL (Table 2.8 and Fig. 2.5). Besides, anemic rats also showed lower tissue non-heme iron levels in liver, spleen, kidney and heart than normal rats as in Experiment 1. The only exception was that the difference in body weight was not significant in Experiment 2. But overall, the results still confirmed that low-iron diet induced iron-deficiency anemia.

Table 2.8 Body weight, hemoglobin concentration and tissue non-heme iron levels (Mean ± SEM) of normal and anemic rats after the acclimation period (Day 14) in Experiment 2

	Normal	Anemic
Body weight (g)	116.2 ± 4.1[a]	103.4 ± 4.8[a]
Hemoglobin (g/dL)	14.0 ± 0.4[a]	5.0 ± 0.1[b]
Liver non-heme iron (μg/g)	42.1 ± 1.3[a]	7.2 ± 0.3[b]
Spleen non-heme iron (μg/g)	58.5 ± 7.9[a]	6.7 ± 0.7[b]
Kidney non-heme iron (μg/g)	17.0 ±0.6[a]	3.2 ± 0.4[b]
Heart non-heme iron (μg/g)	23.0 ± 0.8[a]	8.9 ± 0.1[b]

[a,b] Means with different superscripts within the same row are significantly different ($p<0.05$).

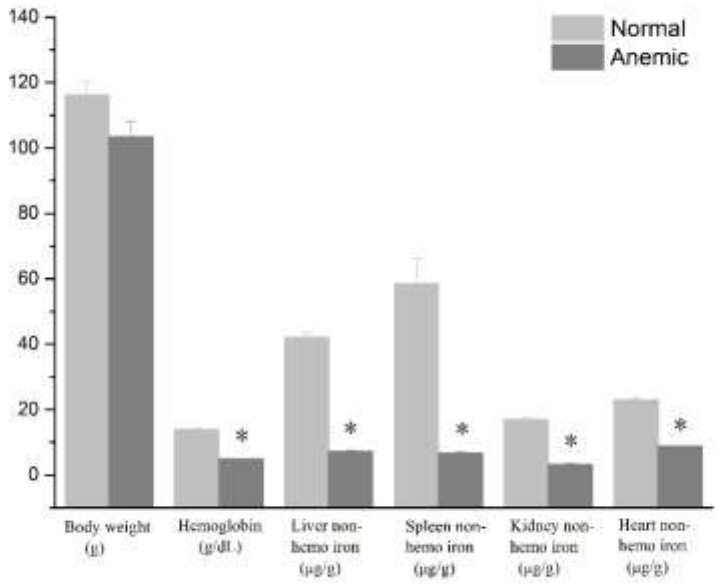


Fig. 2.5 Body weight, hemoglobin concentration and tissue non-heme iron levels (Mean ± SEM) of normal and anemic rats in Experiment 2 on Day 14.
*Significant difference ($p<0.05$) between normal and anemic rats.

2.4.2.2 Weight gain and water intake

During the feeding trial, normal rats gained weight much faster than the anemic rats. In general, all rats gained weight in a linear fashion, although the rate was much slower in anemic rats from Day 22-49 (Fig 2.6). This might be due to the occasional diarrhea observed in some anemic rats during the experiment. Anemic groups supplemented with Synergy1 or inulin had lower body weight when compared to the anemic group without supplementation (Table 2.9) on Day 49 (and also on Day 35 for the Anemic-Synergy1). There were no significant differences

in body weight among other anemic rat groups at any time points or between the 2 normal rat groups (Day 14, Day 21, Day 28, Day 35, Day 42 and Day 49).

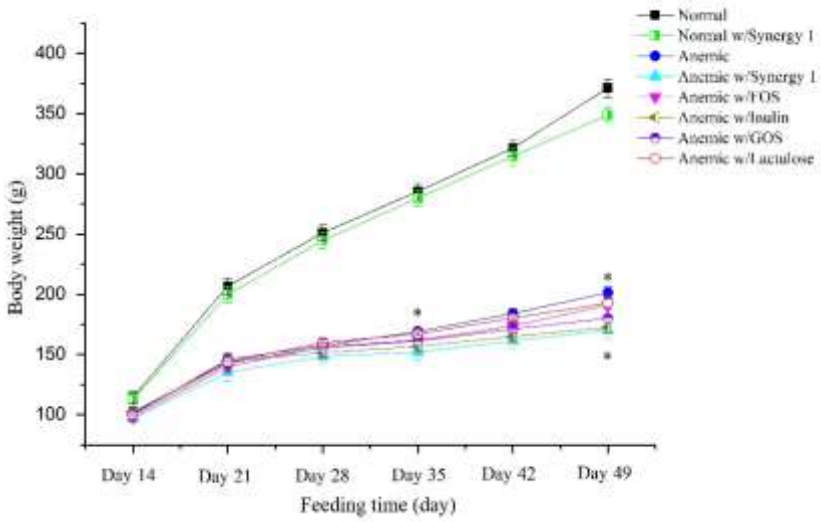


Fig. 2.6 Weekly changes in body weight (g, mean ± SEM) of rats in Experiment 2

Table 2.9 Body weight of rats (g, mean ± SEM) in Experiment 2

Treatment groups	Day 14	Day 21	Day 28	Day 35	Day 42	Day 49
Normal	114.2 ± 5.1	206.6 ± 6.6	251.0 ± 6.5	285.4 ± 6.6	321.5 ± 6.7	371.2 ± 7.6
Normal w/Synergy1	112.6 ± 5.2	199.7 ± 7.0	244.6 ± 6.9	279.9 ± 6.6	314.8 ± 7.6	349.0 ± 6.3
Anemic	101.1 ± 5.0	145.6 ± 5.9	157.4 ± 2.7	169.0 ± 2.6	184.0 ± 3.8	201.3 ± 4.4
Anemic w/Synergy1	96.8 ± 4.6	135.2 ± 7.0	148.3 ± 6.0	151.9 ± 6.5*	161.1 ± 6.5	170.0 ± 6.7*
Anemic w/FOS	97.6 ± 4.1	139.8 ± 4.8	156.2 ± 6.4	161.9 ± 6.6	173.7 ± 6.4	190.8 ± 8.9
Anemic w/inulin	102.2 ± 3.3	144.1 ± 3.7	151.4 ± 4.2	156.7 ± 3.8	164.9 ± 3.6	172.4 ±5.3*
Anemic w/GOS	100.4 ± 4.7	143.0 ± 6.9	155.6 ± 7.8	161.1 ±7.8	171.1 ± 7.3	180.0 ± 8.0
Anemic w/lactulose	100.0 ± 3.7	143.5 ± 5.3	159.7 ± 2.5	167.0 ± 4.4	180.2 ± 3.3	192.6 ± 3.9

* Significant difference ($p<0.05$) when compared to anemic rats without supplementation at the same time points.

The daily water intake in this experiment showed significant differences both between the normal groups and among the anemic groups. The normal rat group with Synergy1 supplementation consumed less water in the last two weeks (Day 36-49) than the normal group without prebiotics (Table 2.10). Among the anemic groups, the groups supplemented with FOS, GOS or lactulose had lower water intake than the one without supplementation (Table 2.10). The lactulose group had the lowest water intake during the whole feeding trial, followed by the FOS group which started to show lower intake from the second week (from Day 22) and the GOS group which started from the third week (from Day 29). Nevertheless, none of these groups showed any significant differences in body weight when compared to rats without supplementation.

In general, prebiotics did not affect the weight gain in normal or anemic rats even if some prebiotics (FOS, GOS and lactulose) caused less water consumption.

Table 2.10 Daily water intake (ml, mean ± SEM) during the feeding trial in Experiment 2

Treatment groups	Day 15-21	Day 22-28	Day 29-35	Day 36-42	Day 43-49	Total Mean
Normal	24.7 ± 0.6	23.7 ± 0.9	26.9 ± 0.7	28.4 ± 0.6	27.0 ± 0.4	26.1 ± 0.6
Normal w/Synergy1	25.8 ± 1.1	26.4 ± 0.8	26.5 ± 0.8	23.3 ± 1.0*	21.5 ± 1.0*	24.7 ± 0.7
Anemic	24.8 ± 0.7	25.2 ± 0.4	26.1 ± 0.2	28.2 ± 1.1	27.3 ± 0.	26.5 ± 0.6
Anemic w/Synergy1	23.1 ± 1.1	24.9 ± 1.1	26.0 ± 1.2	28.1 ± 0.7	26.9 ± 1.6	25.6 ± 1.1
Anemic w/FOS	20.4 ± 0.9	20.6 ± 0.7*	22.7 ± 1.0*	22.5 ± 0.6*	22.8 ± 0.6*	21.5 ± 0.5*
Anemic w/inulin	22.5 ± 0.5	22.4 ± 0.5*	25.8 ± 0.7	25.9 ± 0.5	25.8 ± 0.7	24.3 ± 0.2
Anemic w/GOS	22.2 ± 1.1	21.1 ± 1.1	22.4 ± 0.7*	24.1 ± 0.7*	23.4 ± 0.5*	22.7 ± 0.5*
Anemic w/lactulose	16.3 ± 0.4*	17.8 ± 0.7*	22.7 ± 0.8*	23.8 ± 0.8*	24.3 ± 0.9	21.0 ± 0.6*

* Significant difference ($p<0.05$) when compared to anemic rats without supplementation at the same time points.

2.4.2.3 Hemoglobin concentration and non-heme iron level during feeding trial

As in Experiment 1, anemic rats remaining on the low-iron diet would continue to show low hemoglobin concentration throughout the feeding trial, ranging from 3.7 - 5.8 g/dL (Table 2.11). Also similar to Experiment 1, FOS supplemented anemic rats showed a mild but significant increase ($p<0.05$) in hemoglobin concentration after 4 weeks (Day 42) compared to anemic rats without supplementation (Fig 2.7 and Fig. 2.8). However, anemic rats with FOS

supplementation showed no significant differences in non-heme iron levels in liver and kidney, and a lower level in heart (Table 2.12 and Fig. 2.9).

Other anemic rat groups supplemented with prebiotics (Synergy1, inulin, GOS or lactulose) showed higher values in hemoglobin concentration in general when compared with the anemic group without supplementation (Table 2.11 and Fig. 2.8), but only the GOS group was statistically significant after 3 weeks (Day 35). The GOS group also showed a significant higher non-heme iron level in liver, but similar to FOS, a significant lower non-heme iron level in heart (Table 2.12 and Fig. 2.9). The inulin group also had a lower non-heme iron level in heart. Anemic groups with Synergy1 or lactulose were not significantly different from the anemic group without supplementation.

Table 2.11 Hemoglobin concentration of rats (g/dL, mean ±SEM)

in Experiment 2

Treatment groups	Day 14	Day 21	Day 28	Day 35	Day 42	Day 49
Normal	12.4 ±0.3	14.2 ±0.3	14.1 ±0.3	15.0 ±0.5	15.3 ±0.2	15.6 ±0.2
Normal w/Synergy1	13.1 ±0.4	14.0 ±0.4	14.3 ±0.2	15.1 ±0.3	15.8 ±0.3	15.9 ±0.3
Anemic	5.4 ±0.2	4.4 ±0.1	4.2 ±0.1	3.7 ±0.1	3.7 ±0.1	3.8 ±0.1
Anemic w/Synergy1	5.3 ±0.2	4.7 ±0.1	4.3 ±0.1	3.8 ±0.1	3.6 ±0.1	4.3 ±0.3
Anemic w/FOS	5.5 ±0.1	4.8 ±0.2	4.3 ±0.1	3.9 ±0.1	4.1 ±0.1*	4.3 ±0.1
Anemic w/inulin	5.5 ±0.2	4.8 ±0.1	4.3 ±0.1	3.8 ±0.1	3.7 ±0.1	3.9 ±0.2
Anemic w/GOS	5.8 ±0.1	4.9 ±0.1	4.4 ±0.2	4.3 ±0.1*	4.0 ±0.1	4.1 ±0.1
Anemic w/lactulose	5.3 ±0.2	4.7 ±0.2	4.2 ±0.2	4.1 ±0.1	4.0 ±0.2	3.8 ±0.1

* Significant difference ($p<0.05$) when compared to anemic rats without supplementation at the same time points.

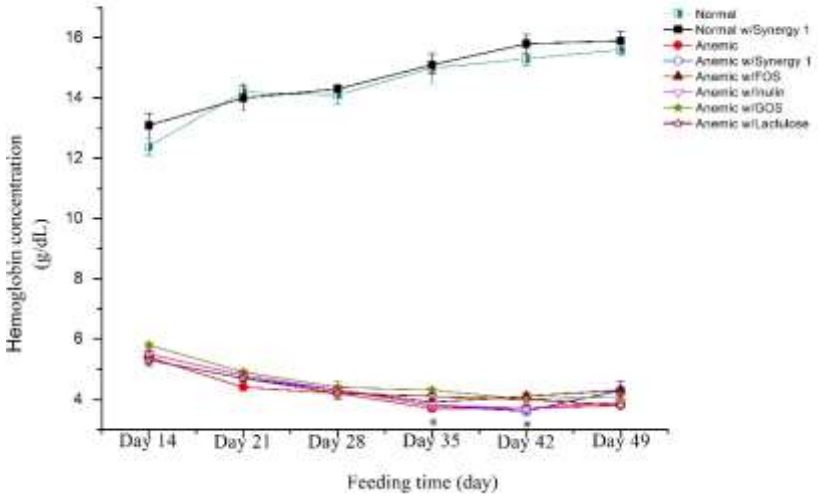


Fig. 2.7 Weekly changes in hemoglobin concentration (g/dL, mean ±SEM) of rats in Experiment 2.

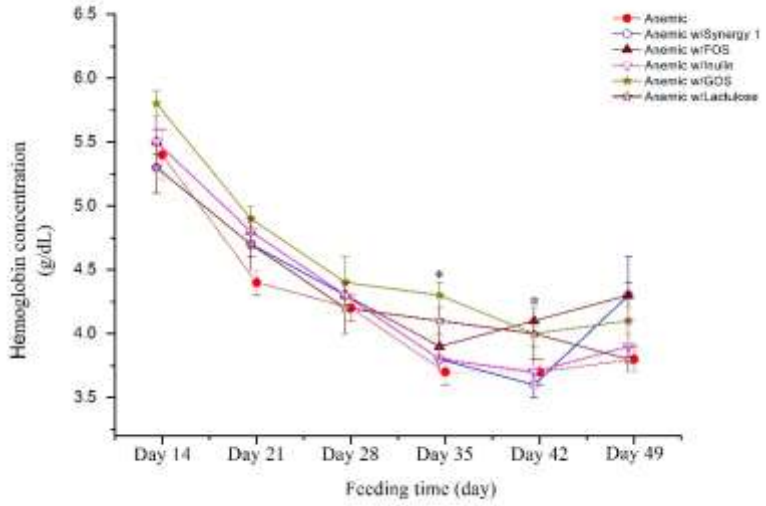


Fig. 2.8 Weekly changes in hemoglobin concentration (g/dL, mean ± SEM) of only anemic rats in Experiment 2. * Significant difference ($p<0.05$) when compared to anemic rats without prebiotics supplementation at the same time points.

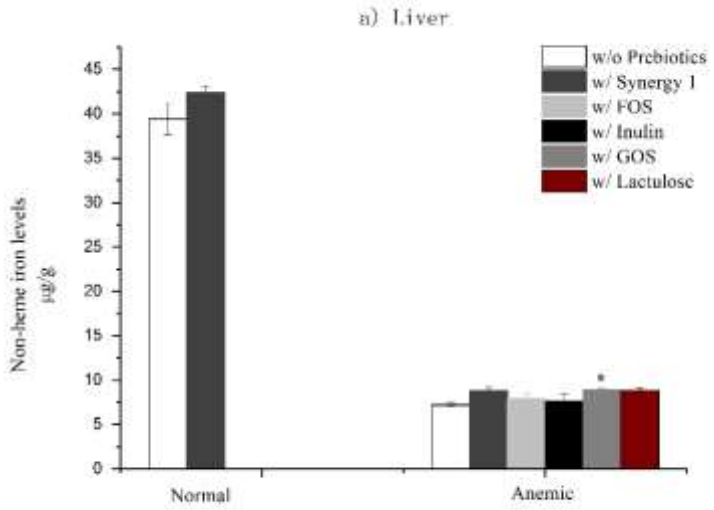
As for the normal groups, only Synergy1 had been studied because Synergy1, a combination of inulin and FOS, had been reported to be more efficient than inulin or FOS alone (Gibson et al., 2010). Our results, however, showed that there were no differences either in hemoglobin concentration (Table 2.11 and Fig. 2.7) or tissue non-heme iron levels and (Table 2.12 and Fig. 2.9).

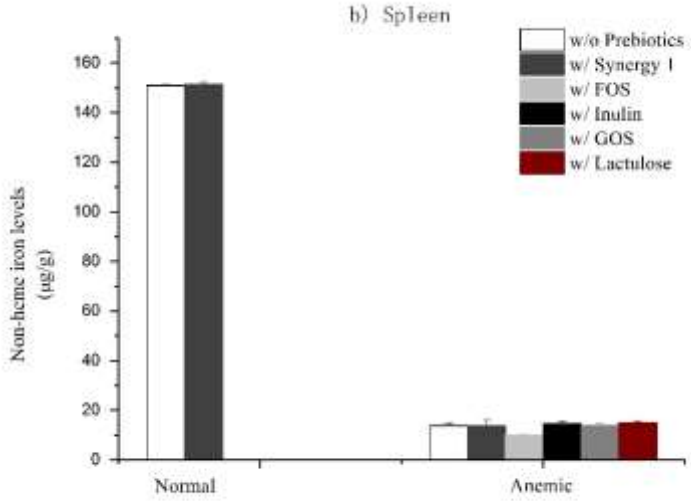
Results from Experiment 2 were mostly consistent with Experiment 1, i.e., FOS supplementation could mildly increase the hemoglobin concentration in anemic rats. In addition, GOS also had a mild enhancing effect on improving the iron status of anemic rats. Other prebiotics including inulin, lactulose and Synergy1 showed no effects on improving iron status in anemic rats. Synergy1 did not affect rats with normal iron status.

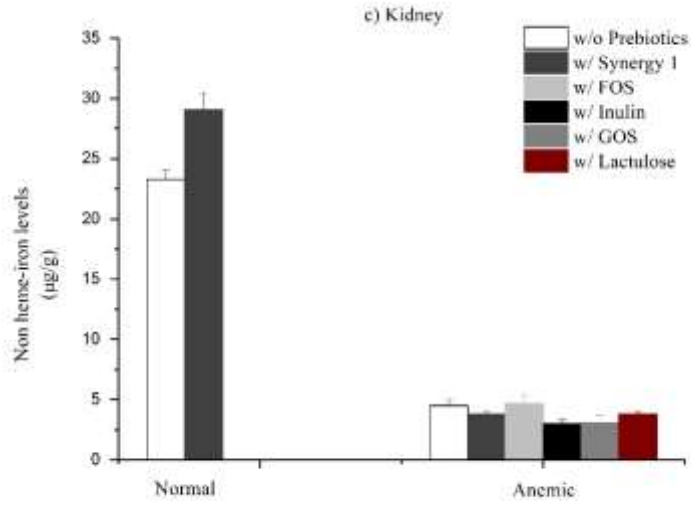
Table 2.12 Rat non-heme iron levels (μg/g, mean ± SEM) after the feeding trial (Day 49) in different tissues in Experiment 2

Treatment groups	Liver	Spleen	Kidney	Heart
Normal	39.4 ± 1.8	151.0 ± 0.4	23.3 ± 0.8	26.4 ± 0.7
Normal w/Synergy1	42.4 ± 0.7	151.5 ± 0.6	29.1 ± 1.4	26.5 ± 0.7
Anemic	7.2 ± 0.2	13.8 ± 1.3	4.5 ± 0.5	16.8 ± 2.1
Anemic w/Synergy1	8.8 ± 0.4	13.7 ± 2.4	3.8 ± 0.2	15.2 ± 0.8
Anemic w/FOS	7.9 ± 0.6	10.1 ± 0.4	4.7 ± 0.7	13.4 ± 0.6*
Anemic w/Inulin	7.6 ± 0.8	14.7 ± 1.0	3.0 ± 0.3	13.8 ± 0.3*
Anemic w/GOS	8.9 ± 0.2*	14.0 ± 1.0	3.1 ± 0.6	13.3 ± 1.0*
Anemic w/lactulose	8.8 ± 0.3	15.0 ± 0.7	3.8 ± 0.2	14.8 ± 0.6

* Significant difference ($p<0.05$) when compared to anemic rats without supplementation at the same time points.


a) Liver
w/o Prebiotics
w/ Synergy 1
w/ FOS
w/ Inulin
w/ GOS
w/ Lactulose
Non-heme iron levels
μg/g
45
40
35
30
25
20
15
10
5
0
Normal
Anemic


b) Spleen
w/o Prebiotics
w/ Synergy 1
w/ FOS
w/ Inulin
w/ GOS
w/ Lactulose
Non-heme iron levels
(μg/g)
160
140
120
100
80
60
40
20
0
Normal
Anemic


c) Kidney
w/o Prebiotics
w/ Synergy 1
w/ FOS
w/ Inulin
w/ GOS
w/ Lactulose
Non heme-iron levels
(μg/g)
35
30
25
20
15
10
5
0
Normal
Anemic

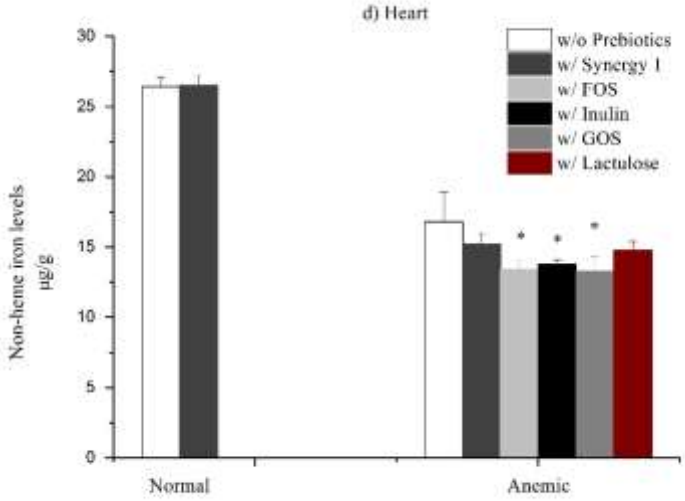


Fig. 2.9 Rat tissue non-heme iron levels (μg/g, mean ± SEM) after the feeding trial (Day 42) in a) liver; b) spleen; c). kidney; d) heart in Experiment 2. *Significant difference ($p<0.05$) when compared to anemic rats without prebiotics supplementation

2.4.2.4 SCFA analysis of rat colon contents

Rat colon contents collected after sacrifice were further analyzed for SCFA composition. As shown in Fig. 2.10, the individual SCFAs (acetic acid, propionic acid, butyric acid, isobutyric acid, pentanoic acid and isopentanoic acid) in the standard mixture were well separated. In addition, the retention time of the internal standard DL-2-methylbutyric acid was 7.001 min, also well separated from the individual SCFAs. These results validated the method and conditions used for SCFA detection.

The correlation coefficient (R^2) of the linear regression equation of each SCFA component in the standard mixture was larger than 0.99 (Table 2.13),

suggesting that the standard curves constructed using 7 different concentrations were reliable. Specifically for the linear regression equation, the X-axis represented the ratio between the concentration of each component in the standard mixture and concentration of the internal standard whereas the Y-axis represented the ratio between the peak area of each component and the peak area of the internal standard.

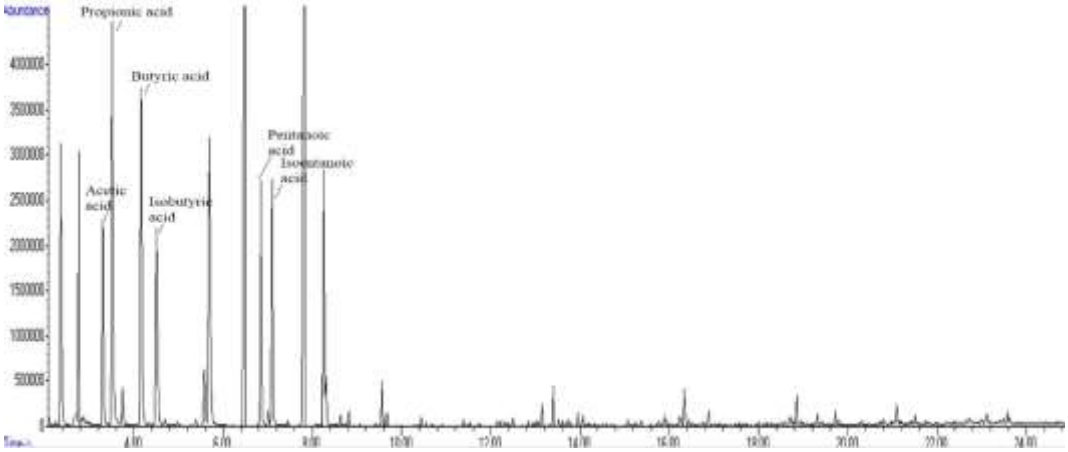


Fig. 2.10 Chromatogram of the SCFA standard mixture

The detection accuracy of each short chain fatty acid component had been calculated by injecting 50 μg/mL standard to GC-MS for six times. The Relative Standard Deviation (RSD) of each component ranged from 0.63% - 1.44% (Table 2.13), indicating favorable accuracy. The Limit of Quantitation (LOQ) of each component was 10-fold of the standard deviation (SD), which was derived and calculated from detection of 0.1 mg/L standard for six times. The LOQ of acetic acid, propionic acid, butyric acid, isobutyric acid, pentanoic acid and isopentanoic acid were 0.06035, 0.00467, 0.00113, 0.03850, 0.05526 and 0.04670, respectively, indicating the GC-MS system is capable of measuring SCFA at low concentrations.

Table 2.13 Retention time, linear regression and accuracy of each component of the SCFA standard mixture

	Retention time (min)	Linear regression equation	Correlation coefficient (R^2)	Linear range of concentration (mg/L)	SD (mmol/L)	RSD (%)
Acetic acid	2.360	Y=8.848x-1.515	0.995352	0.1~500	0.006035	1.447944
Propionic acid	3.512	Y=8.347x-1.577	0.994634	0.1~500	0.000467	0.863728
Butyric acid	4.160	Y=6.871x-0.3488	0.998484	0.1~500	0.000113	1.337596
Isobutyric acid	4.506	Y=4.271x-0.4510	0.995473	0.1~500	0.003850	0.823687
Pentanoic acid	6.846	Y=2.191x-0.07351	0.999181	0.1~500	0.005526	0.639777
Isopentanoic acid	7.098	Y=2.391x-0.2713	0.998744	0.1~500	0.004670	0.982210

After two weeks of acclimation (on Day 14), colon contents from normal and anemic rats were first analyzed. While acetic acid, butyric acid and pentanoic acid of anemic rats were significantly lower than normal rats, propionic acid and isobutyric acid showed no differences between the 2 groups (Table 2.14). Isopentanoic acid in anemic rats was too low to be detected.

Table 2.14 SCFA analysis of rat colon contents (μg/mg, mean ± SEM) after the acclimation period (Day 14) in Experiment 2

Treatment groups	Acetic acid	Propionic acid	Butyric acid	Isobutyric acid	Pentanoic acid	Isopentanoic acid
Normal	510.5 ± 5.5	184.3 ± 1.6	195.4 ± 3.2	104.4 ± 0.5	37.2 ± 0.5	73.3 ± 1.01
Anemic	403.1 ± 8.7*	183.8 ± 5.1	72.2 ± 1.1*	105.5 ± 6.4	21.4 ± 0.2*	-

*Significant differences ($p<0.05$) between the normal and anemic groups.

At the end of the feeding trial (on Day 49), groups supplemented with prebiotics showed significant differences in SCFA concentrations (Table 2.15). For normal rats, the group with Synergy1 had significant higher acetic and propionic, but lower butyric and pentanoic acid concentrations than the group without supplementation. While for anemic rats, the groups supplemented with Synergy1 or inulin were significantly lower in acetic, propionic and butyric acid concentrations than the group without supplementation, with the inulin group showing overall the lowest concentrations in SCFAs. In addition, the groups with FOS and GOS had lower butyric acid concentration, and the group with lactulose had lower propionic and butyric acid concentrations.

Among anemic groups, all groups with prebiotics (except inulin) supplementation showed a significant higher concentration in isobutyric acid, but no differences in pentanoic acid when compared to the group without supplementation (Table 2.15). The isopentanoic acid concentration for all anemic

groups supplemented with prebiotics was too low to be detected. Only FOS increased significantly the concentrations of all three main SCFA products (acetic acid, propionic acid and isobutyric acid) found in the colon contents of anemic rats, when compared to no supplementation.

Table 2.15 SCFA analysis of rat colon contents (μg/mg, mean ± SEM) after the feeding trial (Day 49) in Experiment 2

Treatment groups	Acetic acid	Propionic acid	Butyric acid	Isobutyric acid	Pentanoic acid	Isopentanoic acid
Normal	532.3 ± 5.8	225.8 ± 4.6	321.2 ± 4.1	117.0 ± 2.3	76.9 ± 4.0	91.56 ± 3.67
Normal w/Synergy1	574.8 ± 9.2*	371.0 ± 4.5*	147.4 ± 5.2*	115.7 ± 1.6	22.5 ± 0.2*	88.0 ± 0.2
Anemic	441.2 ± 3.9	227.8 ± 1.1	81.0 ± 0.8	106.8 ± 1.0	20.5 ± 0.1	75.9 ± 0.1
Anemic w/Synergy1	396.1 ± 4.7*	199.1 ± 1.3*	52.5 ± 1.5*	137.3 ± 5.3*	20.7 ± 0.2	-
Anemic w/FOS	514.2 ± 11.1*	243.3 ± 1.9*	65.3 ± 1.7*	135.6 ± 1.3*	20.7 ± 0.1	-
Anemic w/inulin	350.0 ± 6.4*	138.1 ± 1.1*	42.8 ± 0.5*	103.5 ± 1.8	19.6 ± 0.1*	-
Anemic w/GOS	452.3 ± 6.6	224.8 ± 5.7	69.8 ± 2.3*	114.6 ± 3.0*	20.3 ± 0.1	-
Anemic w/lactulose	457.3 ± 8.0	197.9 ± 1.8*	66.9 ± 1.6*	139.6 ± 2.1*	20.3 ± 0.2	-

*Significant difference ($p<0.05$) when compared to rats fed the same iron diet without supplementation.

2.5 Discussion

2.5.1 Animal Experiment 1

Perhaps owing to the difficulties and ethical concerns associated with studies on anemic human subjects, information about the effects of FOS on humans suffering from iron-deficiency anemia is lacking. In Experiment 1, we utilized the rat model to compare the iron status of normal and anemic rats with or without FOS supplementation, and to investigate if FOS has any promotional effects on the recovery from iron-deficiency anemia. The results showed that FOS could mildly enhance the hemoglobin concentration in anemic rats, but FOS had no effects on normal rats, nor provided any additional boost on the recovery from anemia when rats were switched from a low iron diet to the regular diet.

Rats fed the low-iron diet (12 μg Fe/g diet) during the acclimation period achieved a hemoglobin concentration of 5.0 g/dL and a significantly lower body weight (87.0 g vs 101.9 g), as well as lower tissue non-heme iron levels in liver, spleen, kidney and heart, when compared to normal rats. In a recent study on iron-copper interactions, Ha et al. (2016) reported that feeding male Sprague-Dawley rats a diet containing about 12 μg Fe/g diet for 5 weeks led to iron-deficiency anemia in rats with hemoglobin concentration ranging from 4-7 g/dL, and anemic rats also grew slower than controls. The study by Freitas et al. (2012) also showed that two weeks of iron-deficient diet (10 μg Fe/g diet) induced iron deficiency anemia in weaning Wistar rats, with a hemoglobin concentration of

5.3 - 6.0 g/dL. Our results were consistent with these studies and confirmed that rats receiving the low-iron diet achieved iron-deficiency anemia after the acclimation period, thus providing an iron-deficient rat model for the subsequent feeding trial with FOS supplementation.

During feeding trial, body weight between paired groups with or without FOS had no significant differences at any times evaluated, but anemic rats remaining on the low-iron diet gained weight at a slower rate (smaller weekly percent increases) compared to normal rats on the regular diet. This is consistent with the observations reported by Ha et al. (2016).

There are no clear explanations for the lower water intake by Anemic – Low-iron rats with FOS supplementation than rats without during the first week of feeding trial. No significant differences in body weight or subsequent daily water intake between the pair were observed. In other words, dissolving FOS (a soluble dietary fiber) at 5% w/v did not alter the pattern of water consumption for the rats. Interestingly, while anemic rats switched to the regular diet was able to gain weight at about the same rate as normal rats, water intake remained relatively low throughout the feeding trial. This might be partially explained by the observation that rats with perinatal iron-deficiency anemia, despite after normalization of growth and hematology through dietary iron supplementation, exhibited abnormal behavior, impaired brain function, and marginal reduction in general activities in adulthood (Felt et al. 2006). Nevertheless, the underlying physiological basis for the relatively lower water intake warrants further investigations.

During the experiment, rats took in roughly 20 – 40 mL of water daily, and at 5 % w/v, that would be equivalent to an intake of 1 – 2 g FOS per day during the feeding trial. The typical daily feed intake is 15 g/ day for growing rats (National research Council, 1995). This level of FOS intake was significant given the body weight of the rats was only somewhere between 90 - 310 g. Taken together, the level of FOS intake by the rats in this study was very high relative to their body weight and should be adequate to show any enhancing effects, if FOS indeed improves iron status. As an aside, Moshfegh and others (1999) estimated that the average intake of FOS was about 5 g/d in diets of American adults, and the acceptable intake of FOS could be as high as 30 g/d (Coussement 1999). With an assumed body weight of 70 kg for an average American adult, an acceptable FOS intake would be equal to 0.43 g/kg body weight.

In general, more than two-third of body iron is incorporated into hemoglobin, and liver serves as a major storage site for the remaining body iron (Mejia et al. 1979, Andrews, 1999). Our results suggest that FOS supplementation could lead to mild increases in both hemoglobin concentration and liver non-heme iron level in anemic rats with a low iron intake. Products of FOS fermentation by intestinal microflora include acetic, propionic, and butyric acids (Stewart et al. 2008). It has been hypothesized that these acids may enhance iron absorption by decreasing the pH in the colon environment, thereby increasing iron solubility and bioavailability (Yeung et al. 2005a). Rats with gastrectomy-induced anemia fed diets supplemented with non-digestible disaccharides (difructose anhydride III, and

epilactose) have been shown to have increased short-chain fatty acid pools and decreased pH of cecal contents (Shiga et al. 2006, Suzuki et al. 2010). Presumably, any health effects of prebiotics are conferred in the colon through fermentation by beneficial intestinal microflora. Although it has been established that iron is absorbed predominantly in the duodenum and absorption from the proximal colon is less efficient (Bougle et al. 2002), Sakai et al. (2000) showed that dietary FOS alleviated anemia in gastrectomized rats, and the effect was diminished by cecectomy, suggesting that the proximal colon could potentially still be a site of significant iron absorption during iron-deficiency anemia. The results in this study supported that FOS could have a mild effect on improving the iron status of anemic subjects.

Hemoglobin concentrations of anemic rats switched to the regular diet recovered quickly and were comparable to normal rats after only 2 weeks. In this study, FOS supplementation did not provide any additional effect on the recovery as no significant differences were observed between groups with or without FOS in hemoglobin concentration or tissue non-heme iron levels. Ohta et al (1995) indicated that 5% FOS supplementation elevated the apparent iron absorption and improved recovery from anemia in iron deficient rats within the first week. But they also claimed that it was difficult to evaluate the effect of FOS on recovery from anemia in iron deficient rats because iron absorption was known to increase and recovery was very rapid when fed a normal iron diet. Report by Lobo et al. (2014) suggested that the improving effect of FOS supplementation on iron recovery

could be observed within the first week of repletion period in anemic male Wistar rats. And in Freitas et al. study (2012), lower amount of Fe (25 mg Fe/kg diet) was used in order to have better sensitivity to evaluate the effect of FOS, and improving effect on iron absorption and recovery was observed by 10% supplementation. Therefore, FOS may not have an additional effect on recovery when dietary iron is sufficient (as shown in our results), and it is also possible that the iron level even in a normal diet is too high and overwhelms any potential effect on recovery from anemia.

Normal rats with or without FOS had no significant differences in hemoglobin concentrations at any time points, or tissue non-heme iron levels at the end. These results together suggest that when there is an adequate amount of iron in the diet, FOS has no effects on iron status. Conversely, FOS does not appear to negatively affect iron status.

2.5.2 Animal Experiment 2

As in Experiment 1, rats fed with the low-iron diet reached iron-deficiency anemia after two weeks of acclimation, showing a hemoglobin concentration of 5.0 g/dL, as well as lower body weight and tissue non-heme iron levels.

Daily water intake was significantly lower in the normal rat group with Synergy1 in the 4^{th} and 5^{th} weeks, and also in the anemic groups supplemented with FOS, GOS or lactulose during almost the whole feeding trial. Differences in water intake reported by Freitas et al. (2012) showed that the volume of water intake of

rats supplemented with inulin, Synergy1 or FOS was significantly higher than the control, but not different between the groups receiving the prebiotics. In our study, the anemic groups with supplementation of FOS or lactulose actually had lower average water consumption than the other three anemic groups receiving prebiotics, representing a lower total prebiotics intake. There were no significant differences in average water intake among the anemic groups receiving Synergy1, inulin and GOS, nor between the two normal groups with or without Synergy1. In Experiment 2, rats took in roughly 20 – 30 mL of water daily, and at 5 % w/v, it would be equal to an intake of 1 – 1.5 g prebiotics per day during the feeding trial. This level of prebiotics intake was comparable to other published studies (Videla et al. 2001; Ohta et al. 1995; Sakai et al. 2000; Delzenne et al. 1995). The amount of prebiotics supplementation varied a bit in these studies, ranging from 2 – 10% w/w in diet or w/v in drinking water, with a corresponding intake of 1 – 1.6 g prebiotics/day based on calculations from their data on feed consumption or water intake (Videla et al. 2001; Ohta et al. 1995; Sakai et al. 2000; Delzenne et al. 1995).

One possible mechanism for the enhancing effect of FOS observed in our study could be the selective fermentation of prebiotics by microbiota in the large intestine (Roberfroid 1998). It had been showed in human studies that FOS supplementation can significantly stimulate the growth of *Bifidobacteria* and *Lactobacilli*, especially *Bifidobacteria* (Williams et al, 1994; Bouhnik et al, 1999; Gibson et al, 1995). It had also been demonstrated that high prevalence of *Bifidobacteria* and *Lactobacilli* in the colon could be beneficial for human with

low iron status as they might lead to more available iron for the host by restricting the growth of pathogenic bacteria, therefore preventing pathogenic bacteria using dietary iron (Patterson et al, 2008).

The SCFA concentration in the colon content of the FOS anemic group was significantly higher than the non-prebiotic supplemented anemic group, with all three main SCFAs (acetic acid, propionic acid and isobutyric acid) being higher. Rossi et al. (2005) showed that acetate and lactate were the major fermentation products in fecal cultures by investigating 55 *Bifidobacterium* strains on FOS *in vitro*.

When FOS reached the colon, it is hydrolyzed and fermented by the resident microflora, including *Bifidobacteria* and *Lactobacilli*, to produce SCFAs and other organic acids (e.g., lactic acid) (Sundbery, 2011). An accumulation of these acidic compounds could lead to a decline in pH of luminal environment and contents, which might help to enhance iron absorption by reducing iron to its soluble ferrous form, solubilizing iron to its ionized form, and releasing iron from protein-complexes (Sundbery, 2011; Ohta et al, 1995). Propionate has also been shown to form a soluble complex with iron to make iron more absorbable (Bougle et al., 2002). Therefore, the significantly higher propionic acid in the colon content of anemic rats with FOS is supportive of a higher hemoglobin concentration as shown in the results. Besides, it is also believed that the fermentation and production of SCFAs cause morphological, physiological and molecular changes in the intestine, such as the proliferation of epithelia cells

which increases the absorptive area (Scholz-Ahrens et al. 2001). In the study of Kleessen et al. (2003), FOS supplementation led to an increase in intestinal villi and a deeper mucosal crypt in bacterial colonized rats but not in the germ-free group.

The positive effects of FOS and its fermentation are localized in the intestine and the cecum could be of importance. Lower luminal pH in cecum was observed in the study of Lobo et al. (2014) in iron deficient rats fed with FOS with an increase in iron uptake in cecum. In addition, cecal enlargement is commonly associated with dietary fiber fermentation and the phenomenon has been frequently reported in the literature (Campbell et al. 1997; Lobo et al. 2009; Lobo et al. 2011). Presumably, the enlargement is supposed to be proportional to the fermentability of the fiber (Campbell et al. 1997; Lobo et al. 2009; Lobo et al. 2011) and could contribute to increased iron absorption (Hara, Onoshima and Nakagawa, 2010). In the present study, the anemic group with FOS showed both an increase in SCFA production, as well as enlarged cecum (observed during dissection).

While an enhancing effect on rat irons status in anemic groups supplemented with FOS was seen in our results, other anemic groups with other common fructans (inulin and Synergy1) showed no such improvement. Although inulin and FOS are the most studied prebiotics and both had been reported to selectively stimulate *Bifidobacteria* in the gut (Kolida et al., 2007; Gibson et al., 1995), not all inulin derivatives have the same effects on intestinal microflora and may

influence different segments of the large intestine (Roberfroid, 2005). It had been demonstrated that different types of inulin or FOS were processed differently in the digestive tracts, thus affecting different types of microbial populations, leading to different digestive or metabolic impacts, including the types of SCFA produced and pH changes in intestine (Kleessen et al., 2001; Flickinger et al., 2003). As discussed above, the benefit of enhanced mineral absorption may be mainly due to the fermentation and the production of SCFA products by probiotics. The different influences on the intestinal microflora could lead to different effects on absorption. Besides, it had been reported that the structure and chain length of the fructans may also influence the effect on mineral absorption. Short-chain FOS is more preferable to *Bifidobacteria* as substrate for growth because it is more readily fermentable for its lower molecular weight (Gibson and Wang, 1994; Mckellar et al. 1993; Tashiro et al. 1997). Rossi et al (2005) observed that 55 *Bifidobacterium* strains could use FOS as carbon source, but failed to use inulin. Furthermore, while longer chain inulin may need to reach the distal ileum or the cecum for fermentation, FOS can be fermented not only in the large intestine but also to a greater extent in the small intestine (jejunum and ileum) (Patterson et al., 2010). Therefore, FOS could provide a more noticeable effect than inulin on iron absorption due to greater fermentation in different parts of the intestinal tract. Sakai et al. (2000b) reported that 7.5% FOS feeding could increase iron absorption and promote recovery from post-gastrectomy anemia in gastrectomized rats, but 7.5% inulin supplementation did not result in the recovery from

post-gastrectomy anemia. Petry et al (2012) also demonstrated that inulin did not influence fractional iron absorption in women with low iron status.

As for Synergy1, which is a combination of short chain FOS and long chain inulin, the enhancing effect was still not significant as FOS in Experiment 2. Similar results were also observed in Freitas et al. study (2012), when 10% feeding of "high-performance (HP) inulin" or FOS resulted in a significant higher hemoglobin concentration than the control group, but 10% Synergy1 led to lower value than the control group. The Synergy1 had been reported to be more efficient and to have higher activity than FOS or inulin in calcium absorption (Coudray et al, 2003; Griffin et al., 2002; Younes et al., 2001). But this same effect on iron absorption was barely found. Yasuda et al (2006) showed that 4% Synergy1 improved hemoglobin synthesis in young pigs on a corn and soybean meal, but because of the diet they used, the observed effect of Synergy1 could be a co-effect of Synergy1 and soybean oligosaccharides. Soybean oligosaccharides are a candidate prebiotic and had been reported to stimulate *Bifidobacteria* (Saito et al., 1992; Hayakawa et al., 1990). Besides, the enhancing effect of Synergy1 on calcium absorption may lead to an inhibition of iron absorption, as the absorption of calcium and iron was known to be antagonistic (Hallberg et al., 1991). Another possible reason may be that the 5% of Synergy1 in water was not sufficient to generate the same effect as 5% FOS on iron absorption because 5% Synergy1 equals to 2.5% FOS together with 2.5% inulin.

GOS, derived from lactose, is another type of commercial prebiotic with 85% oligo-galactose and some glucose and lactose. Polymerization degree usually is 2-5 (Macfarlane et al. 2006). It showed a significant elevation (16.2%) on hemoglobin concentration on Day 35 and a significant higher liver non-heme iron level. But the main SCFA increases were not seen. Studies on the effect of GOS on mineral absorption were mainly found on calcium and magnesium, seldom on iron. Maawia et al. (2016) demonstrated an enhancing effect of 5% GOS feeding on iron, calcium and magnesium after 3 weeks in rats, but van den Heuvel et al. (1998) showed that 15 g of GOS, inulin or FOS supplementation to healthy man for 3 weeks had no effect on iron or calcium absorption. There is, nevertheless, evidence supporting GOS as a *Bifidobacterium*-promoting substrate, and to selectively stimulate the growth of *Bifidobacteria* and *Lactobacilli*. Rowland and Tanaka (1993) demonstrated the bifidogenic properties of GOS when germ-free rats inoculated with human feces was fed with 5% (w/w) GOS for 4 weeks, resulting in a significant increase in *Bifidobacteria* and *Lactobacilli*. And Chonan et al (2001) showed in their study that the enhancing effect of GOS on calcium and magnesium absorption was suppressed by feeding of the antibiotic neomycin, indicating that the intestinal bacteria were necessary for GOS to have a positive response. In addition, GOS supplementation increases crypt depth and cell density in the proximal and distal colon (Perez-Conesa et al 2007), providing a larger area for absorption. Therefore, GOS could have a similar enhancing effect as FOS on iron absorption in anemic rats.

Different from fructans and GOS, lactulose is a disaccharide consisting of galactose and fructose. In the current study, similar to Synergy1, anemic rats with lactulose supplementation also had higher numerical values in hemoglobin during wk 3 (Day 35) and non-heme iron level in liver, but the results were not statistically significantly when compared to anemic rats with no supplementation. Lactulose had been reported to stimulate *Bifidobacteria* in rats fed with infant formula containing 0.5 – 1% lactulose for 5 weeks without fecal pH change (Nagendra and Venkat Rao, 1992). However, a follow-up study showed no significant differences in absorption and retention of nitrogen, calcium, phosphorus and iron in rats fed with infant formula with or without 0.5 – 1.0% lactulose (Nagendra et al., 1994), but the amount of lactulose used might be too low to generate an obvious effect. An improving effect of lactulose on calcium and magnesium absorption (Brommage et al.1993; Demigne et al., 1989; Heijnen et al., 1993) and decreased cecal and ileum pH (Demigne et al., 1989; Heijnen et al., 1993) had been observed in rats by using higher concentration (5-10%). These studies indicated that lactulose has the potential to enhance on iron absorption. But further investigations are needed, especially its effects on iron absorption and the dosage required.

Though all these 5 prebiotics had been reported to have a positive effect and had the potential to improve iron absorption, only FOS and GOS showed a significant enhancement in Experiment 2. The effect of GOS on hemoglobin improvement in anemic rats was faster and was more obvious than FOS, showing

16.2% increase on Day 35, compared to 10.8% increase on Day 42 for FOS. Owing to their different structures, chain lengths, and compositions, their stimulation on intestinal microflora could be different. GOS did not show differences in SCFA productions but FOS resulted in significant higher acetic, propionic and isobutyric acid concentrations. One can speculate that GOS and FOS affect different microbial strains and at different sites along the intestine. The other three did not have a significant effect on improving anemic status. But Synergy1 and lactulose showed some noticeable results in hemoglobin concentration and liver non-heme iron level. Further studies on these two prebiotics to confirm their enhancing effects (of lack thereof) should be conducted.

Except for the anemic group with inulin, all other prebiotics caused a significant higher isobutyric acid in rats when compared to the group without supplementation. The main SCFAs identified in the current study are acetate, propionate and isobutyrate (or butyrate in normal rats), but the effects of SCFAs on iron absorption and the mechanism involved with the enterocyte need to be further investigated.

2.6 Conclusions

Structures and chain lengths of prebiotics could influence their effects on iron absorption, presumably due to their different interactions with intestinal microflora. The short chain oligosaccharides were more effective than longer chain oligosaccharides in improving iron status in anemic rats based on our results. Both FOS and GOS improve the iron status of anemic rats with a low iron intake. Effect of GOS appears to be more pronounced and faster than FOS. While FOS may not have any additional effects on recovery from anemia in rats already with adequate iron intake, none of the prebiotics shows any adverse effects on iron status. Prebiotics are a sub-category of functional foods and can be added to many foods including yogurts, cereals, breads, biscuits, milk desserts, ice creams, spreads, drinks as well as an animal feeds and supplements. Product developers should consider incorporating FOS and GOS not only in foods and beverages to enhance the consumer appeal, but also in functional food products targeting populations at a higher risk of iron-deficiency anemia.

Chapter 3 Dcytb and DMT-1 expression in Caco-2

3.1 Introduction

Caco-2 cells were originally derived from human colon adenocarcinoma cells (Fogh et al., 1977; Tuomalo and Salminen, 1998) and could spontaneously differentiate into polarized, columnar cells under standard culture conditions about three weeks post confluence. The confluent monolayer formed could express characteristics of mature enterocytes which could represent the small intestinal epithelium in both morphology and function (Yee, 1997; Hilgers et al, 1990; Pinto et al., 1983). These characteristics include expression of brush border enzymes and transporters, exhibition of well-developed microvilli and tight junction which are typical features in normal, transporting epithelium (Beaulieu and Quaroni, 1991). Owing to these differentiation properties, Caco-2 cell line tissue model is widely used as an *in vitro* model for various researches, such as absorption studies of nutrients or drugs, as well as metabolomic studies during absorption in the intestinal mucosa in nutrition or pharmacology (Yee, 1997; Hilgers et al, 1990). Particularly, it has been shown that Caco-2 cell culture is appropriate for study of iron bioavailability and absorption in relation to dietary factors (Ismail, 1999).

The epithelium of the small intestine is a highly dynamic system, being spatially separated into proliferative, differentiating, and functional cells in the lower and upper crypt regions and on the villi, respectively. Most of our knowledge about absorption processes has been derived from experimental animals (Kédinger

et al. 1987, Evans et al. 1994) or human colon cancer cell lines (Rousset 1986, Whitehead and Watson, 1997). Animal models offer limited information about absorption in humans; for example, major differences in intestinal cell differentiation in humans and rodents have been reported (Simon-Assman et al. 1994). In addition, major differences in animal and human intestinal environment, such as luminal pH conditions and leakiness of the small intestine, have also been observed. Therefore, information obtained from experimental animal models cannot always be transposed to human situation.

Besides, *in vivo* studies performed with humans and laboratory animals are expensive, time consuming and at times ethically questionable. Therefore, *in vitro* methods, as accurate as possible, are still commonly used in screening of new drug candidates. Immortalized animal and human cell cultures, often of cancer origin, have been used for estimation and prediction of human drug absorption. Several possible *in vitro* human cell models are available for this purpose, one of which is the Caco-2 cell model, a well characterized cell line. According to Biopharmaceutics Classification System (BCS) and with FDA approval, Caco-2 cells can be used as a screening method for new drug candidates during drug discovery and development (Guidance for Industry, FDA 2000, Artursson and Borchardt 1997, Rubas et al. 1996).

Caco-2 cell line was established from a moderately well differentiated colon adenocarcinoma obtained from a 72-year-old patient (Fogh et al. 1977). Caco-2 cells differentiate spontaneously in culture and exhibit structural and functional

differentiation patterns characteristic of mature enterocytes (Pinto et al. 1983). Caco-2 cells reach confluence within 3-6 days and reach the stationary growth phase after 10 days in culture (Braun et al. 2000). The differentiation is completed within 20 days (Pinto et al. 1983). The differentiated cells exhibit high levels of alkaline phosphatase, sucrase, isomaltase and aminopeptidase activity characteristic to enterocyte brush border microvilli. The structural and functional differentiation of the microvilli is associated with the polarization of the monolayer after confluence. The structural polarity is also apparent from the presence of tight junctions, which are formed during the differentiation. The monolayers exhibit a barrier function as judged by high Trans Epithelial Electric Resistance (TEER) values (200-600 Ω/cm^2, grown on polycarbonate filters) and a minimal permeability of mannitol (m.w. 182 g/mol), Lucifer yellow (453 g/mol), polyethylene glycol (4000 g/mol), inulin (5000 g/mol), and dextran (70000 g/mol) (Hidalgo et al. 1989). Fully differentiated Caco-2 cells form an epithelial membrane with a barrier function similar to the human colon (Artursson et al., 1993) but express carrier proteins similar to the small intestine (Baker and Baker 1992, Hidalgo et al. 1989, Wilson et al. 1990). One of the major disadvantages of the Caco-2 cell model is the lack of the mucus producing goblet cells, which would cause the lack of a prominent mucus layer that is present *in vivo* (Wikman Larhed and Artursson 1995, Hilgendorf et al. 2000). Several studies had been trying to establish and characterise a new co-culture cell model including Caco-2 and HT-29

to overcome this problem (Allen et al., 1991; Walter et al., 1996; Meaney and O'Driscoll, 1999).

As discussed in the previous chapter, FOS has been suggested to have positive effects on improving iron status in anemic rats. In order to have a better understanding of the mechanism involved, two genes relevant to iron regulation, Dcytb and DMT-1, were investigated in this part of the project at both protein and mRNA levels. Dcytb and DMT-1 are involved in non-heme iron absorption, and both play an important role at the absorption site (see Chapter 1). Dcytb is a membrane protein and is highly expressed in the apical membrane of duodenal microvilli. It functions as a ferrireductase in mammals by reducing iron in ferric state to ferrous state before transport into the enterocyte. Besides, Dcytb has been shown to be rapidly regulated by iron deficiency, hypoxia and increased systemic iron requirements (McKie et al., 2001; McKie, 2008). Divalent metal transporter 1 (DMT-1) is a proton-coupled transporter that mediates ferrous iron from intestinal lumen into the enterocyte after the reduction of ferric iron (Gunshin et al., 1997) and its expression is influenced by iron status. DMT-1 has been studied in both animals and human cell cultures, and its isoform 1A and isoform +IRE are responsible for regulating iron status (Mackenzie et al. 2007).

The main objective of this part of the research project was to use the Caco-2 cell culture model to elucidate the mechanism involved in the improvement of iron status by FOS. Effects of FOS and its fermentation products (SCFAs) on the expression of Dcytb and DMT-1 both at protein and mRNA levels were studied.

3.2 Materials

3.2.1 Medium and reagent

Dulbecco's Modified Eagle Medium (DMEM, 500 ml) with 4 mM L-glutamine and 3.7 g/L sodium bicarbonate, and Minimum Essential Medium with Earle's Salts (MEM/EBSS, 500 ml) with 2 mM L-glutamine were purchased from Hyclone (USA).

For medium supplements, fetal bovine serum (FBS) was purchased from Hyclone (New Zealand), Penicillin-streptomycin (pen/strep) antibiotic solution (100X), Phosphate Buffer Solution (PBS) (pH 7.4, no Ca^{2+} and Mg^{2+}) were purchased from Gibco (Hong Kong). HEPES and DAPI (4',6' diamino-2-phenylindole) were from Sigma (China). Vectashield mounting medium for fluorescence was purchased from Vector (USA). Type I Collagen stock solution (3 mg/mL) was purchased from Invitrogen (China). Stripping buffer, 30% Bis/Acrylamide Mix (29:1), and eECL Western Blot kit were purchased from CWBio (China). RIPA buffer was from Beyotime (China). Sodium Dodecyl Sulfate (SDS), Ammonium Persulfate (APS), TEMED Dithiothreitol (DTT), SCFAs (Acetic, propionic and butyric acid) as anhydrous sodium salts were from Sigma (Hong Kong); nonfat milk powder was from Anchor (Hong Kong). Phenylmethanesulfonylfluoride (PMSF) was purchased from MYM biological technology (Japan) and Polyvinylidene fluoride (PVDF) membrane was from GE science (Hong Kong).

3.2.2 Cell line

The Caco-2 cell line was purchased from the Shanghai Cell Bank, Chinese Academy of Sciences (CAS) at passage 23. According to the Shanghai Cell Bank's specifications, the cell was originally from American Type Culture Collection (ATCC).

3.2.3 Cell culture consumables

Sterile centrifuge tubes, disposable plastic sterile pipets and Pasteur droppers, sterile culture flasks, and sterile culture dishes were purchased from Costar (USA). Six-well plates, cell culture dishes and flasks were purchased from Corning (USA).

3.2.4 PCR reagents

BCA Protein Assay kit was purchased from CWBio (China). PrimeScript RT reagent Kit (Perfect Real Time) and SYBR Premix Ex Tap (Tli RNaseH Plus) were purchased from Takara (Japan). Primers were purchased from BGI (China). Sequences of primers (5'-3') were: Actin RNA Forward: TGA CGT GGA CAT CCG CAA AG, Actin RNA Reverse: CTG GAA GGT GGA CAG CGA GG; Dcytb Forward: CAT TCC CGC CAG AAG GTG, Dcytb Reverse: TCC CTG TTC AGT GCC TCC A; DMT-1 Forward: ATG TCA CCG TCA GTA TCC CAA G, DMT-1 Reverse: CTG AGC CGA TGA TAG CCA ACT.

3.3 Methods

3.3.1 Cell maintenance

All cell culture media were heated to 37 ^{o}C in water bath before use. Medium in a seeded T-25 culture flask or culture dish was first aspirated with sterile pipet, followed by washing with sterile PBS. Fresh medium (5 mL) was added to the culture flask or dish and incubated at 37 ^{o}C with 5 % CO_2 and 95 % constant humidity. The medium was changed every two days till confluence.

3.3.2 Subculture of cells (passing cells)

After cells reached confluence, medium was aspirated off from the culture flask or dish, and the cells were washed once by sterile PBS. Half mL of sterile trypsin solution was added to the flask or dish, followed by incubation at 37 ^{o}C for 5-10 min. Cells were detached from the flask or dish by mixing and pipetting up and down with added medium. Detached cells were then sub-cultivated to new flasks or dishes at a ratio of 1:4 to 1:6, and replenished with fresh medium.

3.3.3 Cryopreservation of cells

Freezing medium was prepared and employed to preserve earlier passages of cells in ultralow temperature condition. The components of freezing medium were 10% DMSO, 20% FBS and 70% DMEM. The procedure was similar to the subculture of cells. After confluence, medium was aspirated off from the culture flask or dish, and the cells were washed once by sterile PBS. Sterile trypsin solution

was added, followed by incubation at 37 ^{o}C for 5 min. Cells were detached from the flask or dish by mixing and pipetting up and down with the freezing medium added. A 2-mL aliquot of cell suspension was then transferred to a freezing tube and placed in a -80 ^{o}C freezer overnight. The frozen tube of cells was transferred to a liquid nitrogen tank on the following day.

3.3.4 Resuscitation of cells

When cells were needed to be recovered, a frozen tube from liquid nitrogen tank was first put in a 37 ^{o}C water bath to thaw as quickly as possible. After cells were thawed out, the cell suspension was transferred to a cell culture flask or petri dish with the proper amount of fresh medium added. On the next day, the cells were observed under the inverted microscope and the medium would be replaced with fresh one if cells were attached to the bottom surface.

3.3.5 Cell harvesting

The medium was first aspirated off from the culture dish, followed by adding 0.5-1 mL of sterile trypsin solution, and further incubating at 37 ^{o}C for 5-7 min. When cells were observed to be detached from the dish surface, 5 mL of DMEM was added to neutralize the trypsin. Culture dishes were combined and mixed well, and the content was transferred to 50 mL sterile centrifuge tube for counting.

3.3.6 Cell counting

About 50 μL of cell suspension was transferred to the hemocytometer chambers. The average of 4 areas (1 mm^2 for each area which contains 16 smaller squares) was calculated. The number of cells per mL of the cell suspension could be calculated using the equation:

N x F x 10^4 = amount of cells per mL

(where N is the average counted number of those 4 areas, F is dilution factor if cells were diluted).

3.3.7 Cell seeding

Based on the result of cell counting, correct amount of cell suspension was added to each well to reach 4.75 x 10^5 cells per well in the 6-well plate. Each well was made up to a final volume 2 mL with cell culture medium. Plates were then placed in the incubator (37 ℃, 5% CO_2) and cultured for the required days.

3.3.8 Serum-free MEM treatment medium preparation

For 500 mL of MEM liquid medium, 2.5 mg of insulin dissolved in 2 mL of acetic-dH_2O at pH 2 was added to MEM medium for a final concentration of 5 μg/mL. PIPES, Hydrocortisone, $NaSeO_3$ and Epidermal growth factor (EGF) were then added to MEM for a final concentration of 10 mM, 4 μg/mL, 5 μg/mL, and 10 μg/mL, respectively. Next, 0.85 mL of Tri-iodothyronine (T3) stock solution (20

μg/mL) was added to a final concentration of 0.034 μg/mL. All supplements were made into solution and filter sterilized before being added to the medium.

3.3.9 Gene expression analysis using the 6-well plate design

The design shown in Fig. 3.1 was used for the 6-well plate experiment. Cells were seeded in collagen-coated 6-well plates at a density of 5 x 10^4 cells/cm^2 and maintained to 14 days post confluence for the experiment. On the day of the experiment, growth medium was aspirated off from each well, and the cells were rinsed 3 times with 0.5 mL of serum-free MEM at 37 ^oC. Half mL of the treatment medium was then added into each well. The plates were placed in incubator at 37 ℃ with 5% CO_2 and 95% constant humidity. Expression of iron regulatory genes by cells was quantified at the 0-, 2-, 4-, 6-, 12- and 24- hr time points. On each experiment day, six 6-well plates of cells were prepared simultaneously and one 6-well plate was used for each time point.

The concentration of FOS used was 310 mg/L of medium, and this concentration was selected based on the assumption of daily intake value of 10 g for non-digestible oligosaccharides. FeCl3 as the source of supplemental iron was prepared at 20 μM, 50 μM and 100 μM. For testing the effect of the fermentation products, a mixture of SCFAs (acetic, propionic and butyric) was used. The ratio of each acid used was according to Macfarlane's study (2003). Acetic: propionic: butyric values were 52:20:20, but the total concentration of acids would be equal to 310 mg/L of medium. The original concentration of iron in the medium was

determined by inductively coupled plasma atomic emission spectrometry (ICP-AES).

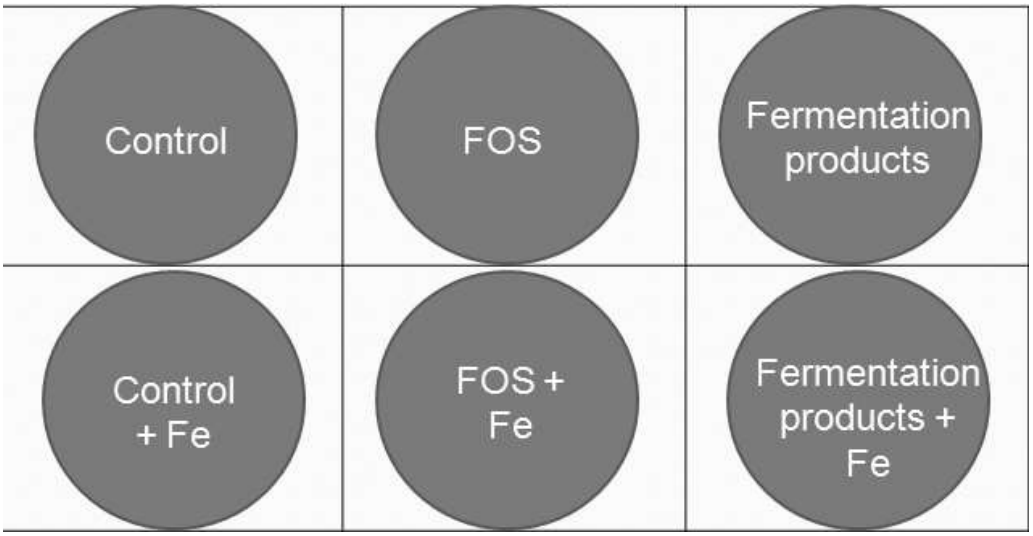


Fig. 3.1 Experimental design using the 6-well plate (Control: MEM without FOS or Fe, Fermentation products: mixture of SCFAs, Fe: in the form of ferric chloride)

3.3.10 Protein sample preparation

In order to avoid protein degradation, the whole process of protein sample preparation was conducted on ice. Medium was aspirated off from each well of the 6-well plate, and cells were rinsed by PBS once. Protease inhibitors, including sodium orthovanadate (Na_3VO_4), sodium fluoride (NaF), EDTA and leupeptin were already included in the commercial RIPA buffer (Beyotime), and PMSF was freshly added at a final concentration of 1 mM in the RIPA buffer before used. Each well was treated with 150 μL of RIPA buffer and left on ice for about 5 min for cell lysis. Then the content in each well was transferred to a 1.5 mL centrifuge tube and

centrifuged at 14,000 g for 5 min. Supernatant was transferred to a new centrifuge tube and cell pellet was also kept as sample. Loading buffer (2X) was added to each sample and mixed well and all samples were put into dry heater for 3 min at 95 ℃.

3.3.11 SDS-polyacrylamide gel preparation

Glass plates were cleaned with ethanol and assembled in the casting stand. The separating (10%) and stacking (5%) gel solutions were prepared separately according to their respective recipes (see below), except for APS and TEMED. APS and TEMED were added to the separating solution, which was then immediately pipetted into the assembled glass plates, filled to a level 1.5-2.0 cm below the front small glass plate. A thin layer of distilled water was added to help polymerization and remove air bubbles. After polymerization (about 30 min), water was poured off, and filter paper was used to remove water residue. Similarly, APS and TEMED were added to the stacking gel solution which was then immediately pipetted to above the separating gel to fill to the top edge of small glass plate. The gel comb was added and the gel was allowed to polymerize for about 15 min.

Recipe for 10 mL of 10% separating gel solution includes 4.0 mL H_2O, 3.3 mL 30% acrylamide mix, 2.5 mL 1.5 M Tris (pH 8.8), 0.1 mL 10% SDS, 0.1 mL 10% APS and 0.004 mL TEMED.

Recipe for 3 mL of 5% stacking gel solution include 2.1 mL H_2O, 0.5 mL 30% acrylamide mix, 0.38 mL 1 M Tris (pH 6.8), 0.03 mL 10% SDS, 0.03 mL 10% APS and 0.003 mL TEMED.

3.3.12 Electrophoresis

The acrylamide gel was placed in electrode assembly, and into the tank, filled with 1X Gel Tank Buffer inside and outside of the electrode assembly. The small glass plate (lower side) was faced to the interior side of electrode assembly. The comb was removed and the molecular weight marker and samples were loaded. A constant current and 80 V was run for first 15 min until the samples arrived at the separating gel. The voltage was adjusted to 100 V or 110 V for the rest of time, until the loading buffer reached the bottom edge of the separating gel.

3.3.13 Wet blotting (Transferring protein from gel to membrane)

After electrophoresis, gel was carefully removed from glass plates with tweezers. The stacking gel was cut off and discarded. The remaining gel was placed in a plastic box containing transfer buffer for about 10 min. The Polyvinylidene difluoride (PVDF) membrane was put into a plastic box containing 100% methanol for 10 min before use. For each gel, 1 piece of PVDF membrane and 2 sheets of thick filter paper were prepared. Next, a gel sandwich was prepared from anode to cathode in the following sequence: fiber pad, thick filter paper, PVDF membrane, gel, thick filter paper, fiber pad. The whole gel sandwich was pressed with a roller to eliminate air bubbles. The gel sandwich was put into the transfer module tank with a frozen unit added, and the tank was filled with transfer buffer to the “blotting” mark. The unit was run at constant voltage, 30 mA overnight or 280 mA for 1 hr. PVDF membrane was stained with Ponceau S (red colour) to insure protein transfer.

Excess red colour was rinsed off with running water and the membrane was kept in blocking solution or TBST.

3.3.14 Western blot

The membrane was blocked in TBST with 5% not-fat milk overnight at 4 ℃ or 1-2 hours at room temperature, followed by washing with TBST for three times (5 min each). The membrane was incubated with the primary antibody at a suitable dilution in TBST indicated by the supplier for 1 hr at room temperature, followed by washing again with TBST for three more times (10 min each). The incubation and washing procedures were repeated with the secondary antibody. The blot was drained and put onto a clear plastic dish, with appropriate amount of ECL substrate added to the membrane. The membrane was covered carefully with plastic wrap and avoided air bubbles. The membrane was then exposed by Bio-rad ChemiDoc Touch Imaging System.

3.3.15 Membrane stripping

After exposure, the membrane was washed in TBST for about 3 min. TBST was poured off and 4 mL of commercial stripping buffer was added, followed by shaking for 15 to 30 min. After being washed for three more times with TBST, the membrane would be applicable for another protein's detection.

3.3.16 RNA sample preparation

In order to avoid degradation, whole process of sample preparation was conducted on ice. Medium was aspirated off from each well of the 6-well plate, and cells were rinsed by PBS once. Each well was added with 1 mL ice cold PBS and cells were scraped off the plate. All contents in each well were mixed well and equal volume was transferred to two 1.5 mL centrifuge tubes, centrifuged at 11,000 rpm for 3 min under 4 ℃, and liquid was removed from tube afterwards. One of the tubes was treated with 0.5 mL Trizol reagent for RNA isolation, the other one was treated with 50 μL RIPA buffer to prepare protein sample for Western blot. RNA samples were kept at -80 ℃ until use. The protein concentrations of samples were measured by commercial BCA Protein Assay kit following the manufacturer's instructions. After measurement, samples were treated with 2X loading buffer, mixed, and put into dry heater for 3 min at 95 ℃.

3.3.17 RNA isolation

RNA samples were taken out from -80 ℃ freezer, thawed on ice, and incubated for 5 min at room temperature. One-tenth mL of chloroform was then added to the sample tube and shaken vigorously for 15 s and incubated 15 min at room temperature. The tube was then centrifuged at 12,000 rpm for 15 min at 4 ℃. After centrifugation, the aqueous phase was transferred to a fresh tube with 250 μL isopropanol added, incubated for 10 min at room temperature and then centrifuged again at 12,000 rpm for 10 min at 4 ℃. RNA was precipitated as pellet at the bottom

of the tube. One mL of ice cold 75% ethanol was used to wash the RNA pellet, and the pellet was collected and dried for 10 min by air before 20 µL DEPC treated water was added to dissolve RNA.

3.3.18 cDNA synthesis

cDNA was synthesized by PrimeScript RT reagent Kit (Perfect Real Time), following the manufacturer's instructions. Briefly, 2 µL of 5x PrimeScript RT Master Mix, 5 µL Total RNA and 3 µL RNase free dH_2O were added to a PCR tube for each sample (total volume was 10 µL), then followed by cDNA synthesis reactions. The conditions used were as follow: 37 ℃ for 15 min, 85 ℃ for 5 s and 4 ℃ for 5 s. cDNA was stored at -20 ℃.

3.3.19 Real time PCR

The PCR system was operated according to manufacturer's instructions: 10 µL SYBR premix Ex Tap (2x), 0.4 µL PCR Forward Primer and 0.4 µL PCR Reverse Primer, 0.4 µL ROX Reference Dye (50x), 4 µL DNA template and 4.8 µl RNase free dH_2O were added to a total volume of 20 µL. Amplification conditions were 94 ℃ for 30 s, followed by 40 cycles (from 94 ℃ for 5 s to 60 ℃ for 35 s).

3.3.20 Mycoplasma contamination test

The DAPI staining method was used for mycoplasma contamination testing. First, a small round glass coverslip was added to each of the well in the 6-well plate before seeding. When cells grew to 80% confluence, the culture media in the well

was discarded and 1-2 mL of PBS was used to wash the cells. Second, 10% formaldehyde was added to each well for 20 min for fixation. The formaldehyde was then discarded, and each well was washed by PBS for 5 min. DAPI was added to a final concentration of 0.1 μg/mL with black cover and PBS was used to wash the cells for the third time for 5 min. The coverslip, on which the cells had been grown, was then taken out and allowed to dry in air. Third, a small drop of mounting medium was added onto a glass slide, and the coverslip was put onto the slide (the side with cells was put on the mounting side). The coverslip with the cells was observed under fluorescence microscope (Leika, DFC 450 C) for any signs of mycoplasma.

3.3.21 Peptide competition test

According to the concentration of antibody used in the western blotting experiment, Dcytb or DMT-1 antibody was diluted in 6 mL blocking buffer (the same buffer used in western blotting experiment). Each diluted antibody was further divided into two tubes with the same volume and the tubes were labeled carefully, i.e. four tubes in total with Dcytb (2 tubes) and DMT-1 (2 tubes).

The blocking peptide was added to one of each antibody's (Dcytb or DMT-1) tube at a ratio of 10:1. The tubes were then labeled "Blocked". For the second tube of each antibody (Dcytb or DMT-1), an equivalent amount of PBS was added and the tubes were labeled "Control". PBS was added to ensure both "Blocked" and

"Control" had the same volume. All tubes were mixed gently and incubated at room temperature for 1hr or at 4 ℃ overnight.

Tow membranes with identical samples were prepared with one membrane used with the "Blocked" antibody and the other with the "Control" antibody. After the western blotting procedures, the membranes were exposed and the results were compared between the blocked and unblocked antibodies.

3.3.22 Statistical analysis

All statistical analyses were done using IBM SPSS Statistics (Statistical Product and Service Solutions, version 21.0). Differences in protein and mRNA levels among multiple treatments were analyzed by one-way ANOVA and Post Hoc comparisons. Differences between only two treatments were analyzed by t-test. Values were given as Mean ± SEM. A p-value of <0.05 was considered significant.

3.4 Results

3.4.1 Verification for analysis

3.4.1.1 Mycoplasma detection

Fig. 3.2 shows the fluorescence image of DAPI staining of Caco-2 cells; cell nuclei could be seen clearly. The area around each nucleus was clear in dark and did not show any small spots. For mycoplasma contaminated cells, as shown in Fig. 3.3, a large number of small, bright, morphologically uniform fluorescent bodies could be seen in the extra-nuclear and intercellular space. Mycoplasma-negative cells (non-contaminated) showed uniform darkness in extra-nuclear background (Chen, 1977).

The Caco-2 cell line was frequently used for studies of dietary iron absorption and bioavailability (Ismail, 1999; Chicault et al., 2005; Bédrine-Ferran et al., 2004; McKie et al., 2001; Riedel et al., 1995; Ramalingam et al., 2000). Healthy, non-contaminated cells are the primary factor for the success of research projects. There are various problems associated with cell lines, but one of the most common concerns is contamination with microorganisms, especially mycoplasma, since mycoplasma is too miniscule to be seen and causes no other obvious changes (Drexler and Uphoff 2002). Unhealthy cells could not proliferate appropriately and die off prematurely, and therefore could not provide reliable experimental conditions. Besides, infection by mycoplasma alters cellular characteristics, and interferes virtually every measurable parameter in experimental investigations or in

routine cultivation. (Drexler and Uphoff 2002; Van Kuppeveld et al., 1994). Thus, contamination check should always be conducted to ensure the quality of Caco-2 experiments before further investigation. Based on the results shown in Fig 3.2, the Caco-2 cell line used in this research was healthy and should be able to provide a reliable cell culture model for subsequent gene expression studies.

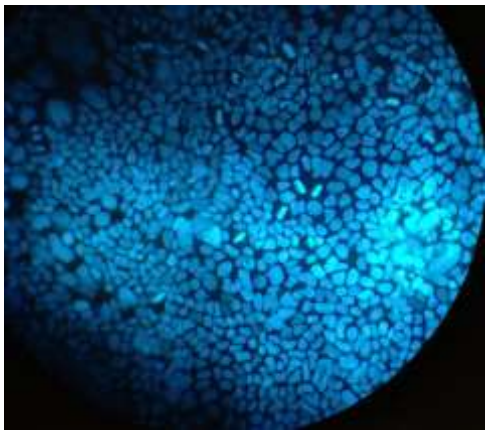


Fig. 3.2 DAPI staining of Caco-2 (Fluorescence microscope 100x)

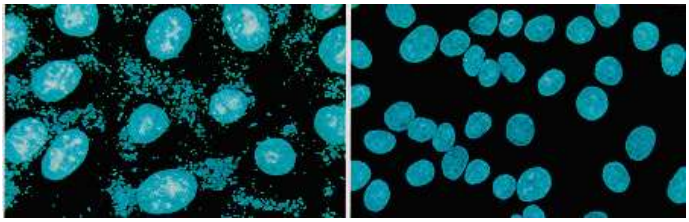


Fig. 3.3 DNA staining of cells, positive for mycoplasma (left) and negative for mycoplasma (right) (Lundin and Lincoln, 1992)

3.4.1.2 Differentiation study

Figure 3.4 and 3.5 showed the results of WB analysis of Caco-2 differentiation. The intensity of each band showed in film was analyzed by Image J software. The expression of Dcytb was normalized by using the house-keeping gene actin. For example, in the 21-day sample, both Dcytb and actin showed strong band intensities (Fig. 3.4), but the ratio was not as high as Day 14 (Fig. 3.5). It could be seen that from day 0 to day 4 post confluence, the expressions of the two proteins were similar, suggesting that the expression of Dcytb did not increase. And the ratio of day 4 was lower than day 0 and 1, which implied a slight decrease in protein expression. This observation was consistent with the report by Ekmekcioglu et al. (1998), in which Dcytb was found to be high in seeded cells, but decline in proliferating cells and only to rise again in differentiated cells. The increment in the expression of Dcytb became more obvious from day 7, and was more notable in day 14 and 21. The protein ratios for day 14 and 21 were 1.8670 and 1.6557 respectively (Table 3.1).

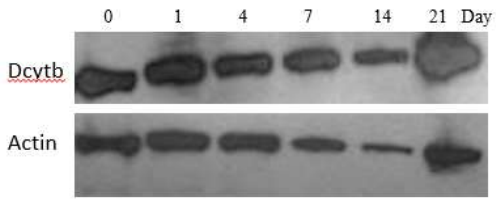
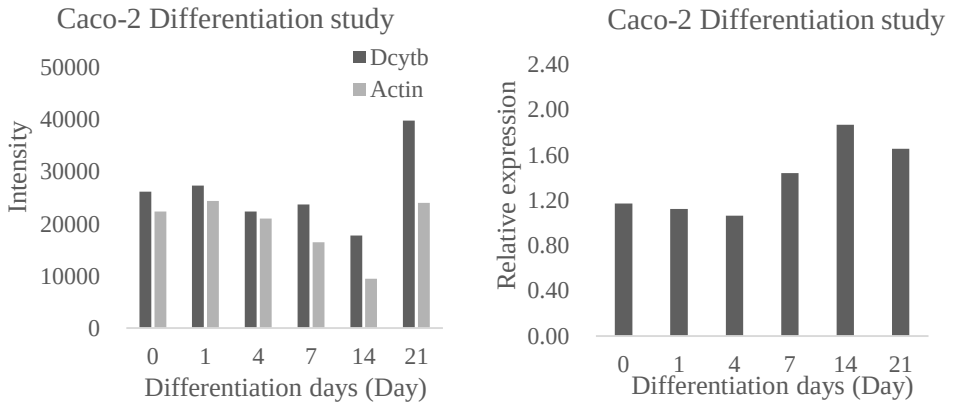


Fig. 3.4 Western results of Dcytb protein expression at 0, 1, 4, 7, 14 and 21 days



A. **B.**

Fig. 3.5 Protein expression in Differentiation study: A. Intensity of Dcytb and Actin in WB analysis for Caco-2 differentiation; B. Relative expression of Dcytb

Table 3.1 Intensity and relative expression of Dcytb in differentiation study

Protein \ Day	0	1	4	7	14	21
Dcytb	26163.15	27358.00	22360.57	23734.47	17736.15	39800.81
Actin	22358.00	24399.28	21049.76	16501.52	9499.74	24038.76
Ratio	1.1702	1.1213	1.0623	1.4383	1.8670	1.6557

Caco-2 cell differentiation appeared to follow a time schedule to express morphological and biochemical characteristics of the absorptive enterocytes (Sambuy et al., 2005), and full differentiation days were reported differently in the literature for different studies: 13 days (Han et al., 1999), 13-14 days (Sandbery, 2010; Scheers and Sandberg, 2008) post seeding or 18 days (Engle et al., 1998; Chicault et al., 2006), 18-21 days, (Halleux and Schneider, 1994) and 21 days post

confluence (Olejnik et al., 2003). The number of days required for Caco-2 cells to be completely differentiated appears to be lab specific. It is therefore important to identify the conditions for cell differentiation specifically in this research project for reliable and repeatable results, as the days for differentiation were not always consistent, and expression of some enterocyte proteins, transporters, and differentiation markers might also not occur in a synchronized manner (Engle et al., 1998). Our results showed that day 14 post-confluence would be most appropriate for Caco-2 experiments. Reports by Ferruzza et al. (2012) and Sandbery (2010) suggested that 14 – 21 days post seeding yielded similar results. Therefore, 14 days post confluence was chosen as the condition for all remaining experiments in this research project.

3.4.1.3 Blocking test

As mentioned in Section 3.2.21, the blocking peptide could bind to the antibody of Dcytb or DMT-1, and thus blocking the protein to be combined with antibody and exposed in western analysis. This test was used to confirm the specificity of antibody and the position of protein band. Although the bands in the block parts were still visible, the bands were weaker and the intensity of bands decreased to 65% in Dcytb (Fig. 3.6 and Table 3.2) and 65-83% in DMT-1 (Fig. 3.7 and Table 3.3) when compared to the controls. The molecular weights of Dcytb and DMT-1 were around 32 KDa and 60-75 KDa respectively. And these were consistent with reports in the literature (Latunde-Dada et al., 2008; Koch et al.,

2003; Chung et al., 2009), confirming that the bands were indeed our target proteins.

Fig. 3.6 Western analysis of Dcytb in blocking test

Table 3.2 Intensity of Dcytb in blocking analysis

Intensity	control	block
1	1,978,342.78	1,285,997.30
2	1,961,359.57	1,290,032.48

Fig. 3.7 Western analysis of DMT-1 in blocking test

Table 3.3 Intensity of DMT-1 in blocking analysis

Intensity	control	block
1	2,291,551.75	1,499,336.00
2	5,215,847.00	4,345,119.83

3.4.1.4 Primer specificity and efficiency

As shown in Fig. 3.8 to Fig. 3.10, all primers presented one peak in the melting curve, meaning that there were no other products or dimmers being formed and the specificity of primers was good. Efficiency of primers was between 90% to 110% (Figure 3.11), which could be considered acceptable (Wei and Yang, 2013). Based on these results, the primers and amplification conditions could be used for gene expression analysis by RT-PCR. The ΔΔ Ct method was used for gene expression analysis where $\Delta\Delta Ct = (Ct_{target} - Ct_{reference})_{sample} - (Ct_{target} - Ct_{reference})_{calibrator}$, and the Relative quantity value = $2^{-\Delta\Delta Ct}$. Actin mRNA expression was used as reference and the control sample mRNA expression as calibrator.

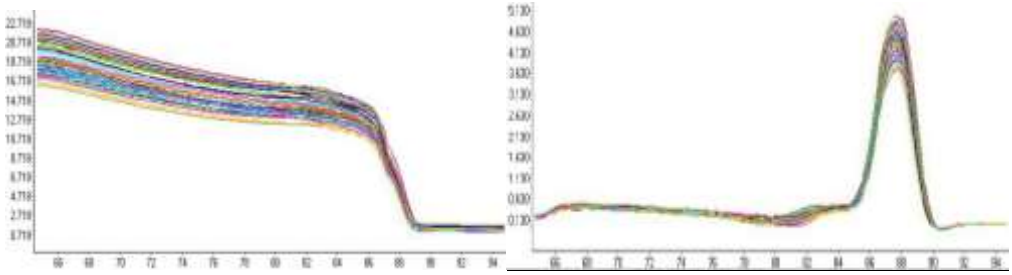


Fig. 3. 8 Melting curve of actin mRNA (fluorescence vs. temperature)

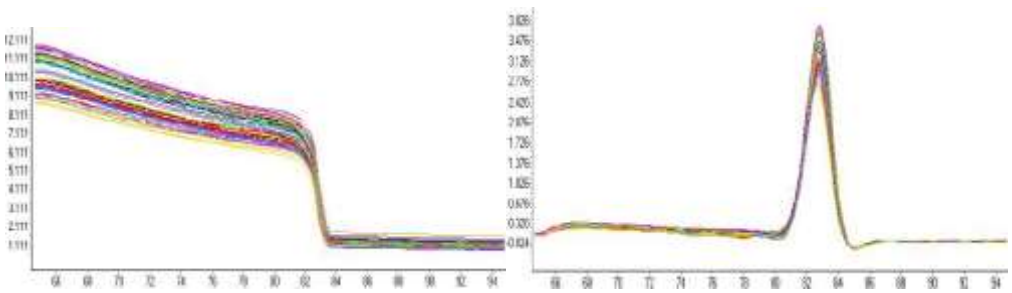


Fig. 3. 9 Melting curve of Dcytb mRNA (fluorescence vs. temperature)

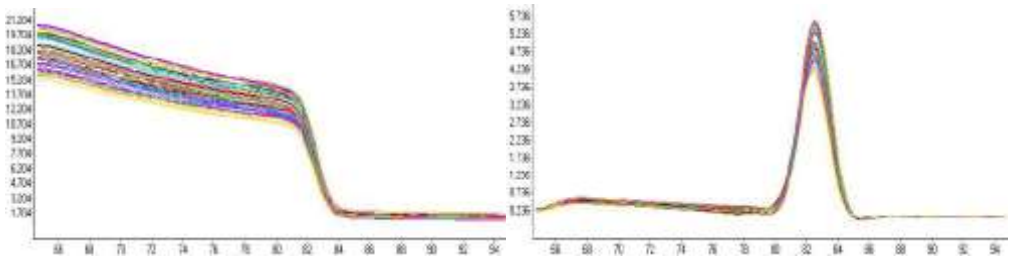
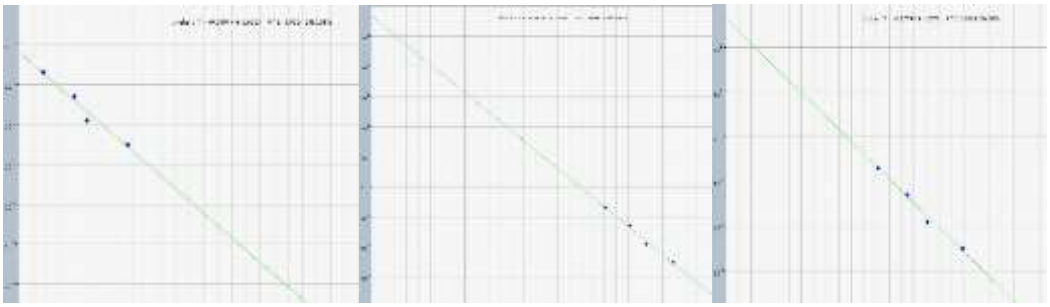


Fig. 3. 10 Melting curve of DMT 1 mRNA (fluorescence vs. temperature)



A **B** **C**

Fig. 3.11 Standard curve and efficiency of primers: A. actin; B. Dcytb; C. DMT-1 (Ct vs. concentration)

3.4.2 Dcytb and DMT-1 expression

Dcytb and DMT-1 expression was investigated at both protein and mRNA levels. Results were expressed relatively using actin as the reference. All mRNA expression used 0 hr as control for normalization. In addition to FOS and SCFAs, effects of different concentrations of iron at 20 μM, 50 μM and 100 μM were also studied.

3.4.2.1 Dcytb expression

As showed in Fig. 3.12, the expression of Dcytb at both protein level and mRNA level were consistent in the control. There were no significant differences at different time points in protein or mRNA. These results were expected as no treatment was given in the control; gene expression in Caco-2 cells should not have changed significantly.

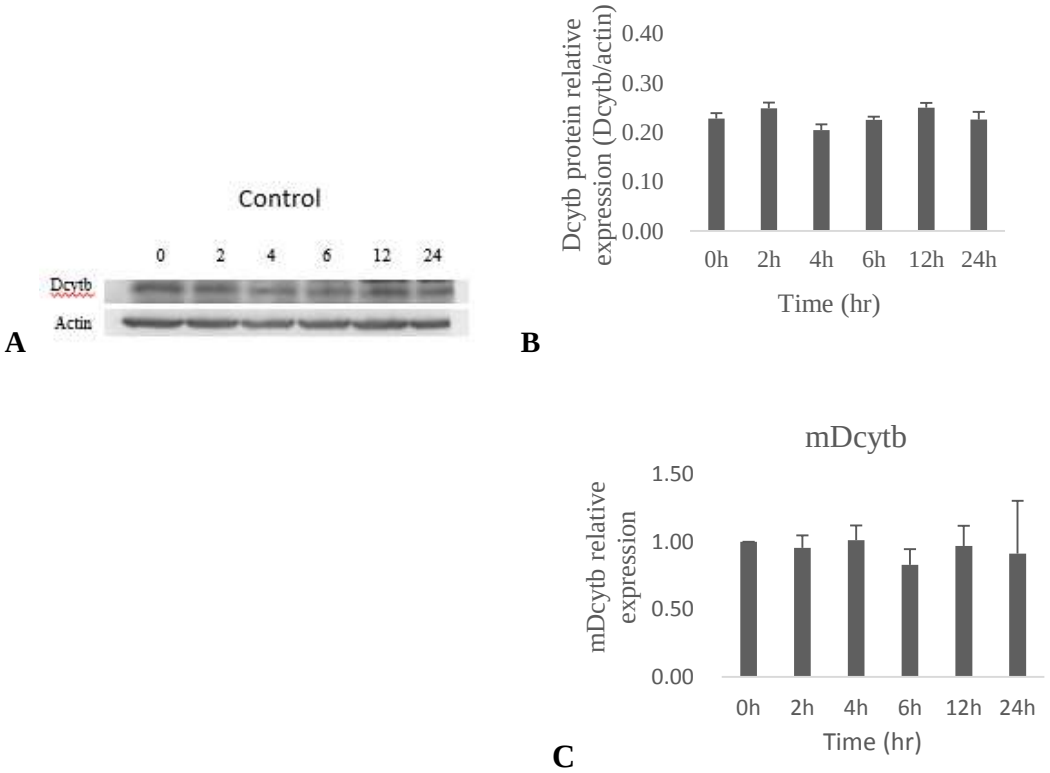


Fig. 3.12 Expression of Dcytb in control at different time points: A. western bands; B. Dcytb protein relative expression; C. Dcytb mRNA relative expression

In Fig. 3.13, there were also no notable changes in Dcytb protein expression when cells were treated with FOS, except a slight increase at 2 hr. There were decreases at 6 and 24 hr for mRNA expression, but the differences were not

significant. These results indicated that FOS treatment did not significantly change Dcytb expression either at protein level or mRNA level.

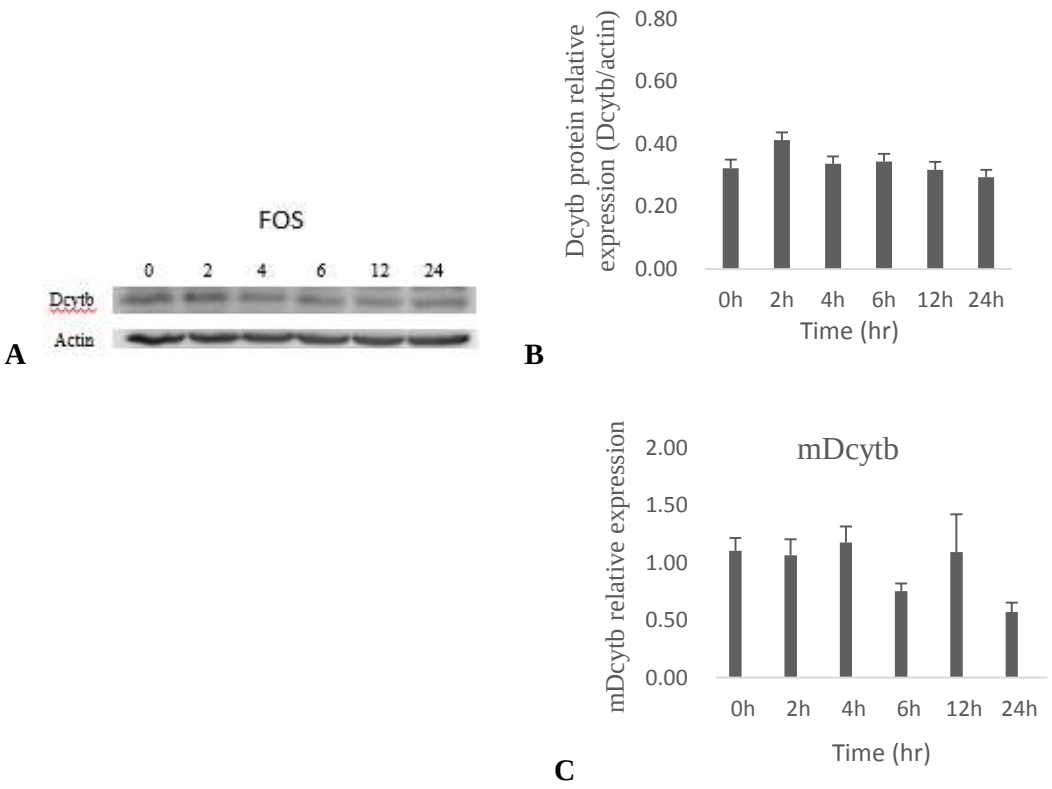


Fig. 3. 13 Expression of Dcytb in cells treated with FOS at different time points: A. western bands; B. Dcytb protein relative expression; C. Dcytb mRNA relative expression

Fig. 3.14 showed that the SCFA treatment increased the Dcytb expression at both protein and mRNA levels, and the differences were significant. The enhancing effect on Dcytb protein was seen after 2 hr and lasted till 12 hr. But the elevated protein level at 2 hr was declining gradually thereafter, and after 24 hr, the expression was not different from the 0 hr time point. For the mRNA level, all time points were significantly higher than 0 hr. Similar to the protein level, after 24 hr,

the expression of Dcytb mRNA went down, but was still significantly higher than the 0 hr time point. These results illustrated that the SCFA treatment could significantly increase Dcytb expression both at protein level and mRNA level after 2 hr, but the effect would diminish after 24 hr.

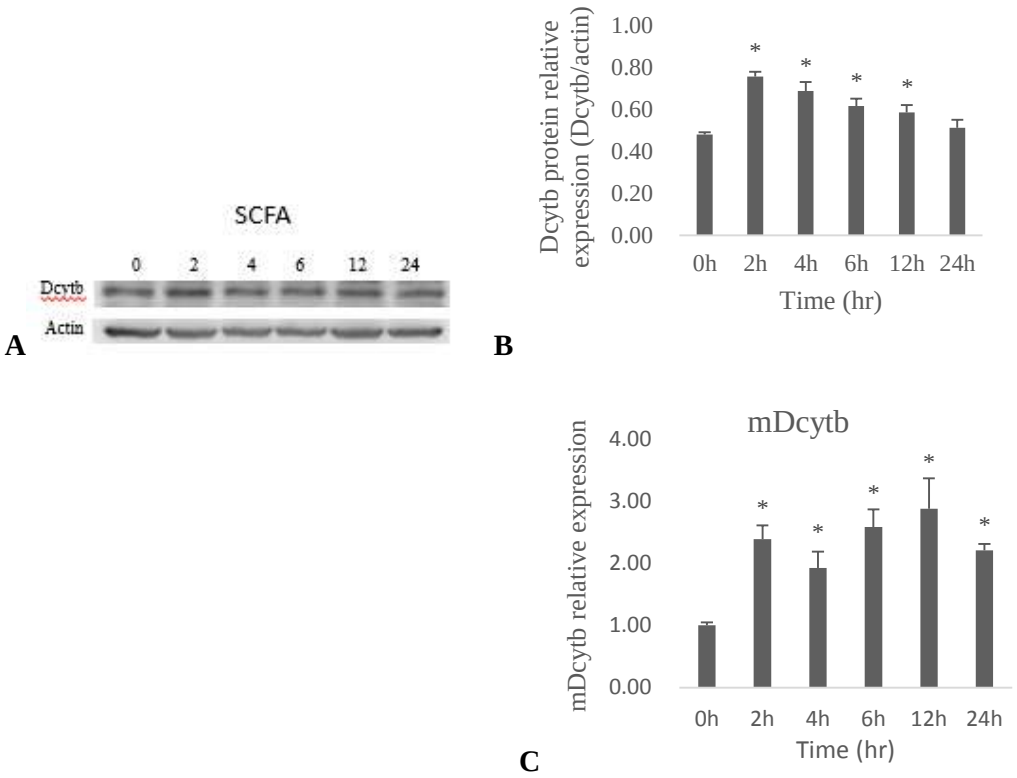
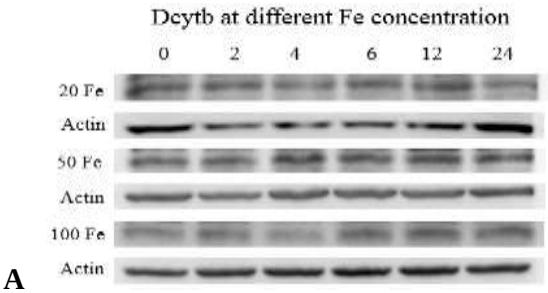


Fig. 3. 14 Expression of Dcytb in cell treated with SCFA at different time points: A. western bands; B. Dcytb protein relative expression; C. Dcytb mRNA relative expression (* Means significantly different compared to the 0 hr time point, $p<0.05$)

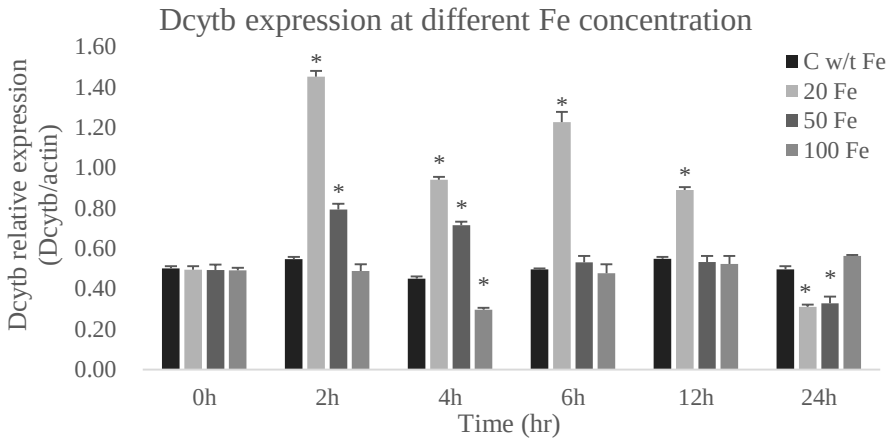
When iron was added to the Caco-2 cells, the expression of Dcytb responded differently according to the concentration of iron. For Dcytb protein, low iron concentration (20 μM Fe) could promote Dcytb expression after 2 hr and expression decreased after 24 hr. At 50 μM Fe, Dcytb protein expression increased

significantly after 2 and 4 hr but also decreased after 24 hr. However, at 100 μM Fe there were no increases in protein expression but a significant decrease at 4 hr.

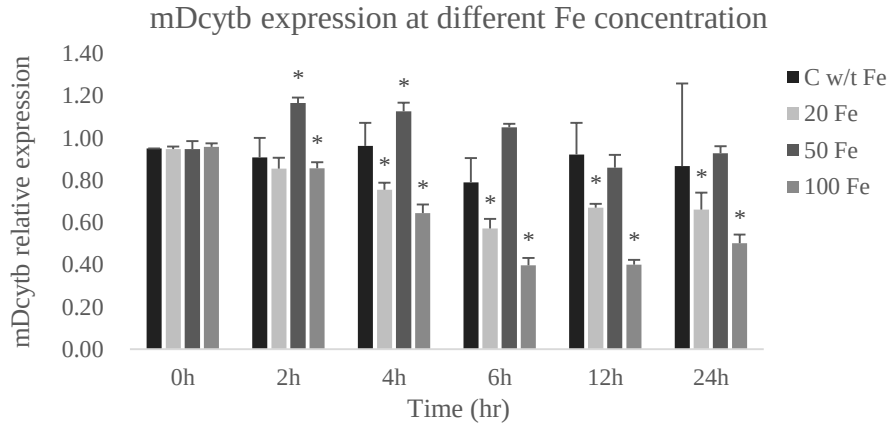
The effect of iron at the mRNA level was different from that at the protein level. At 20 μM and 100 μM Fe, Dcytb mRNA level was down-regulated after 4 hr and 2 hr, respectively. On the contrary, 50 μM Fe increased Dcytb mRNA level after 2 hr. Similar to Dcytb protein level, the increases were significant at 2 and 4 hr, and the elevated effect started to decline after 6 hr. The whole trends of Dcytb expression at protein and mRNA levels were similar at 50 μM Fe supplementation. Overall, iron could affect the Dcytb expression, with lower iron concentration (20 or 50μM) enhances, while high iron concentration (100μM) down-regulates Dcytb expression.



A



B

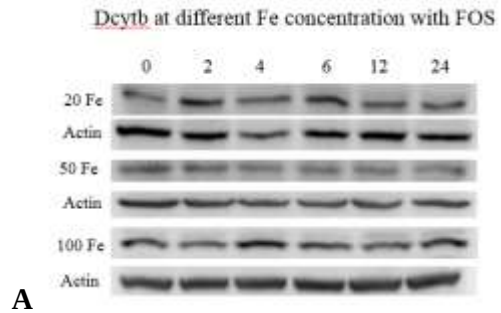


C

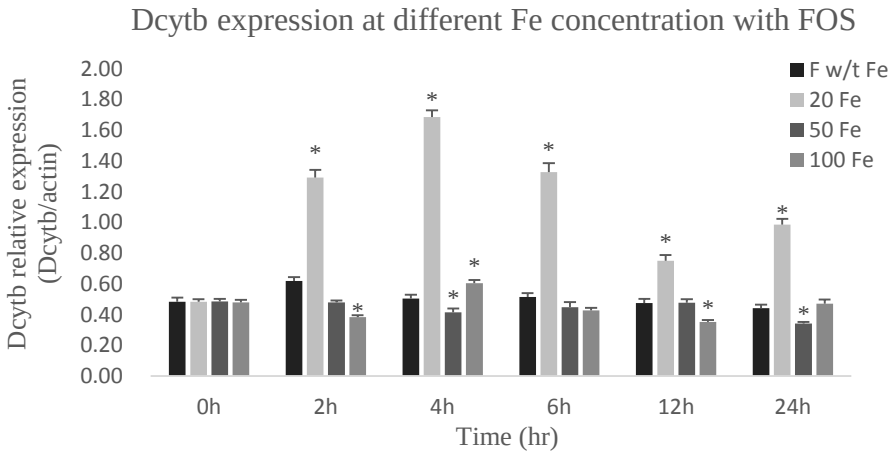
Fig. 3.15 Expression of Dcytb in cells treated with at 20 μM, 50 μM and 100 μM Fe at different time points: A. western bands; B. Dcytb protein relative expression; C. Dcytb mRNA relative expression (* Means significantly different compared to the 0 hr time point, $p<0.05$)

As shown in Fig. 3.16, at the protein level, FOS with 20 μM Fe could still enhance Dcytb expression. When compared to 20 μM Fe alone, the effect with FOS was similar at 2 hr, but greater at 4 hr (Fig. 3.17). FOS with 50 μM Fe did not further enhance the expression, and there was a significant decrease at the 4 and 24 hr time points. FOS with 100 μM Fe also showed a somewhat similar pattern as FOS with 50 μM Fe, but with significant decreases at 2 and 12 hr. Interestingly, an increase also can be seen in 100μM Fe with FOS at 4 hr.

For mRNA, FOS with 20 μM Fe caused a decrease at all time points, and FOS with 50 μM Fe had no obvious effects on Dcytb mRNA expression except a decrease at 6 hr. But FOS with 100 μM Fe had an enhancing effect on Dcytb mRNA expression, which may suggest that FOS could help to increase the Dcytb mRNA expression under higher Fe concentration. Overall when cells were supplemented with FOS together with iron, Dcytb expression could be changed with a positive effect (protein) at 20 μM Fe, but no or little effects at 50 μM Fe. High Fe concentration (100 μM) with FOS increased mRNA level, but not protein level.



A



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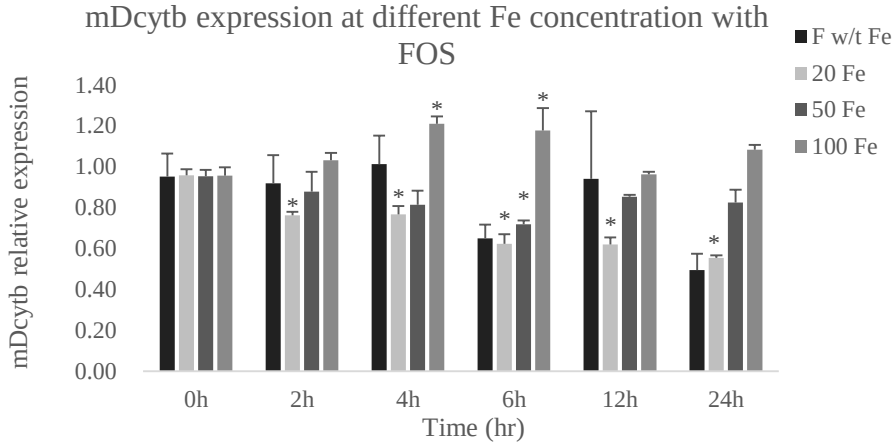


C

Fig. 3. 16 Expression of Dcytb in cells treated with FOS together with 20 μM, 50 μM and 100 μM Fe at different time points: A. western bands; B. Dcytb protein relative expression; C. Dcytb mRNA relative expression (* Means significantly different compared to the 0 hr time point, $p<0.05$)

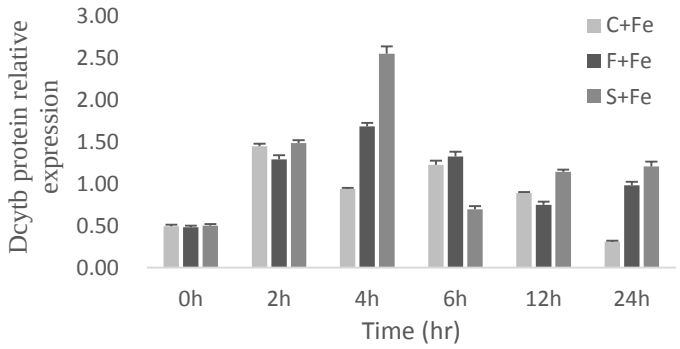


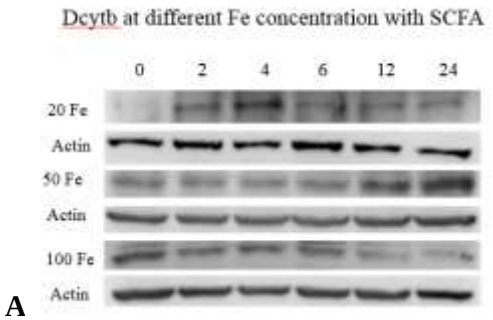
Fig. 3.17 Protein expression of Dcytb in cells treated with FOS or SCFA together with 20 μM Fe (C = Control; F = FOS; S = SCFA)

Protein expression of Dcytb in cells increased when supplemented with SCFA without iron and with 20 μM Fe (Fig. 3.18). The increases were more pronounced with 20 μM Fe, and the combined effect of SCFA + 20 μM Fe on Dcytb protein expression was comparable to FOS + 20 μM Fe or with iron only (C + 20 μM Fe) at 2 hr (Fig. 3.17), but much higher at 4 hr. At 50 μM Fe with SCFA, there were no effects at 2 and 4 hr, but an increasing trend emerged after 6 hr (Fig. 3.18). Nevertheless, the positive effects of SCFA with 50 μM Fe at 6, 12 and 24 hr were still not as strong as SCFA with 20 μM Fe. When cells were supplemented with SCFA with higher Fe (100 μM), Dcytb protein expression was down-regulated.

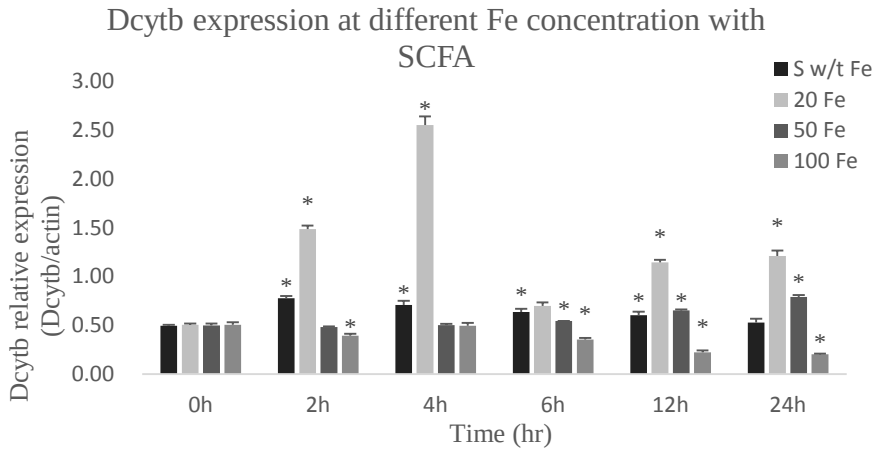
At the mRNA level, SCFA without iron had the strongest effect on increasing Dcytb expression. SCFA with iron, regardless of concentration, showed effects that were not comparable to SCFA without iron. But among the treatments with iron, SCFA with 100 μM Fe up-regulated Dcytb mRNA, and after 24 hr, the mRNA level increased to the same extent as SCFA without iron. At 20 μM and 50 μM Fe, there

were mild increases at some time points (4 hr for 20 μM; and 2, 4, 12 and 24 hr for 50 μM), but none of these increases were as pronounced as SCFA without iron.

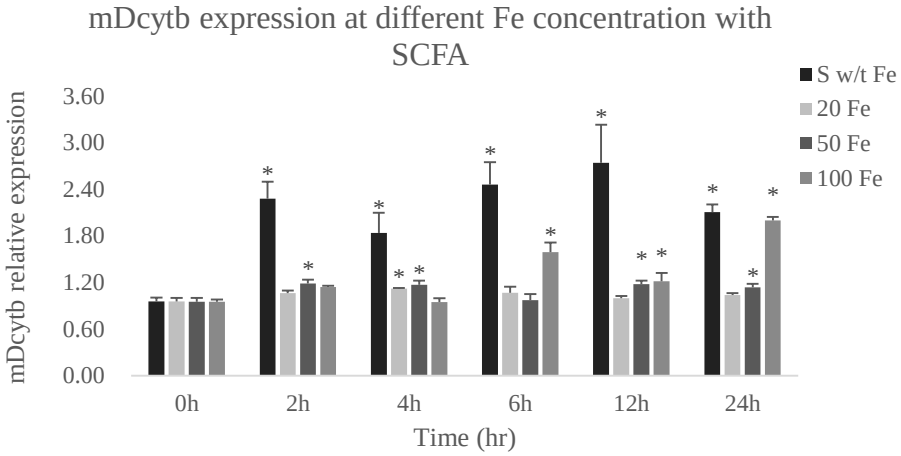
Overall, our results showed that SCFA + 20 μM Fe had the strongest effect on Dcytb protein expression, while SCFA without iron had the strongest effect on mRNA expression.



A



B



C

Fig. 3.18 Expression of Dcytb in cells treated with SCFA together with 20 μM, 50 μM and 100 μM Fe at different time points: A. western bands; B. Dcytb protein relative expression; C. Dcytb mRNA relative expression (* Means significantly different compared to the 0 hr time point, $p<0.05$)

3.4.2.2 DMT-1 expression

Consistent with the results of Dcytb (Fig. 3.12), there were no significant changes in DMT-1 protein or mRNA expression in the control at different time points (Fig. 3.19).

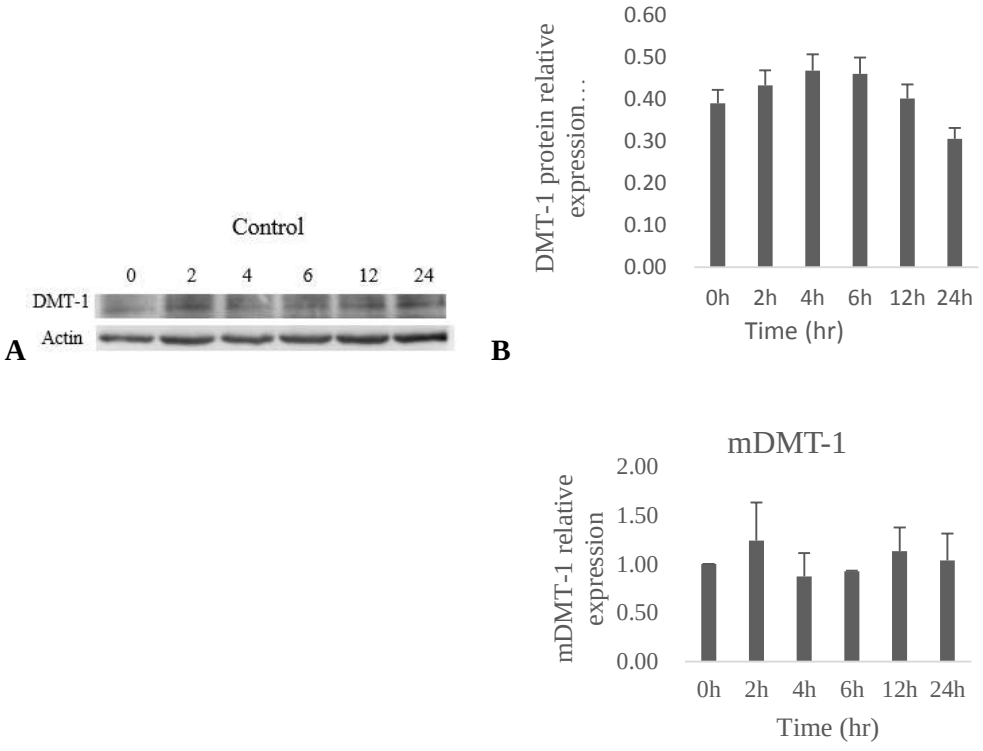


Fig. 3.19 Expression of DMT-1 in control at different time points: A. western bands; B. DMT-1 protein relative expression; C. DMT-1 mRNA relative expression

As shown in Fig. 3.20, FOS significant increased DMT-1 protein expression after 6 hr and the protein expression increased further after 12 and 24 hr. However, there were no significant differences in DMT-1 mRNA at any time points.

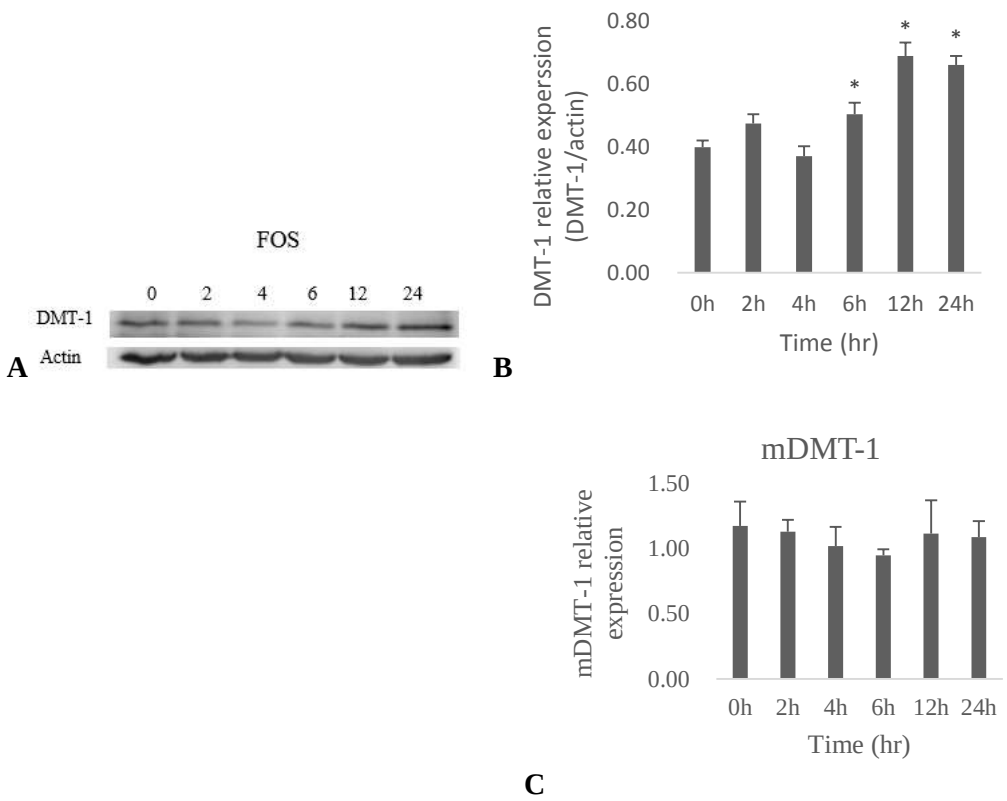


Fig. 3.20 Expression of DMT-1 in cells treated with FOS at different time points: A. western bands; B. DMT-1 protein relative expression; C. DMT-1 mRNA relative expression (*Means significantly different compared to the 0 hr time point, $p<0.05$)

When cells were treated with SCFA, DMT-1 expression changed at both protein and mRNA levels (Fig. 3.21). SCFA increased the DMT-1 protein expression after 2 hr and remained elevated until 12 hr. DMT-1 protein dropped after 24 hr back to the same level as 0 hr. The protein expression at 2 hr was the highest among different time points. For DMT-1 mRNA, DMT-1 expression also increased after 2 hr, but reached an even higher level after 6 hr. Similar to DMT-1 protein, mRNA dropped after 24 hr, but it was still significantly higher than 0 hr. In

short, SCFA can up-regulate DMT-1 expression at both protein and mRNA levels after 2 hr of exposure, and the effect would diminish after 24 hr.

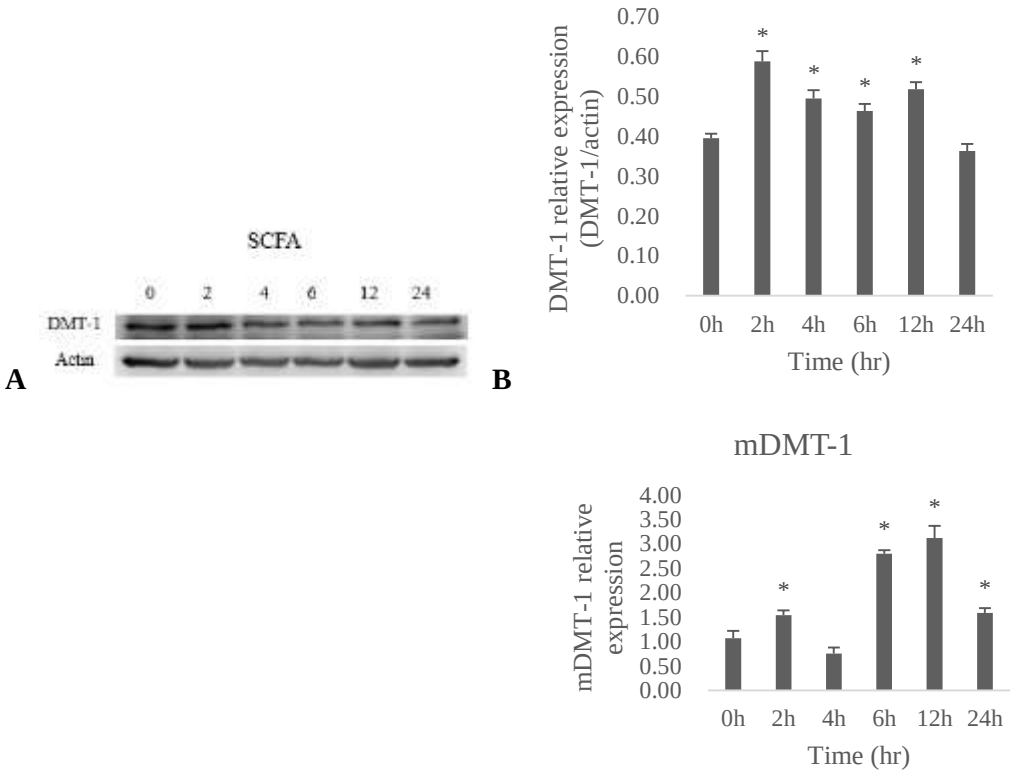
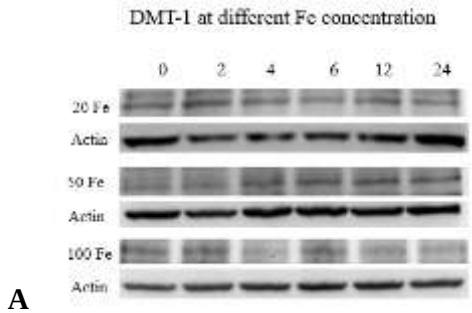


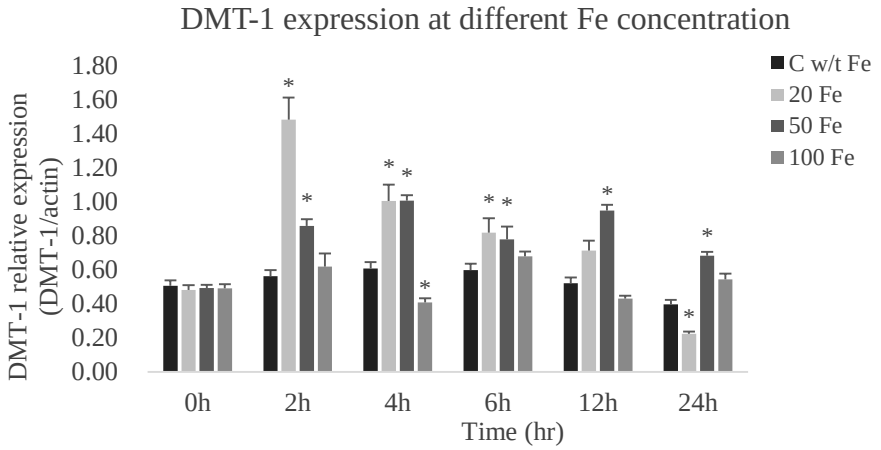
Fig. 3.21 Expression of DMT-1 in cells treated with SCFA at different time points: A. western bands; B. DMT-1 protein relative expression; C. DMT-1 mRNA relative expression (*Means significantly different compared to the 0 hr time point, $p<0.05$)

High concentration of iron (100 μM) provided no enhancing effect on DMT-1 protein expression, and in fact, decreased the expression at 4 hr (Fig. 3.22). However, at 20 μM and 50 μM Fe, increases in DMT-1 protein were noted at 2, 4 and 6 hr. The effect was most obvious after 2 hr with 20 μM Fe. At the 4 and 6 hr time points, 50 μM Fe produced comparable protein levels as 20 μM Fe, but at 12 and 24 hr, 50 μM Fe produced the highest level.

For DMT-1 mRNA level, there were no increases in expression regardless of iron concentration, except only at 24 hr when 50 and 100 μM Fe produced higher DMT-1 mRNA levels (Fig. 3.22). Either low (20 μM) or high (100 μM) concentration of iron significantly decreased the expression after 4 hr or 2 hr, respectively. The negative effect of 20 μM Fe on mRNA expression continued to 24 hr. These results were similar to Dcytb under the same iron treatments, with the main difference being that 20 μM Fe was more effective on Dcytb protein expression whereas 50 μM was more effective on DMT-1 protein expression. Besides, 50 μM Fe was also effective on Dcytb mRNA expression, but only enhanced DMT-1 mRNA level after 24 hr.



A



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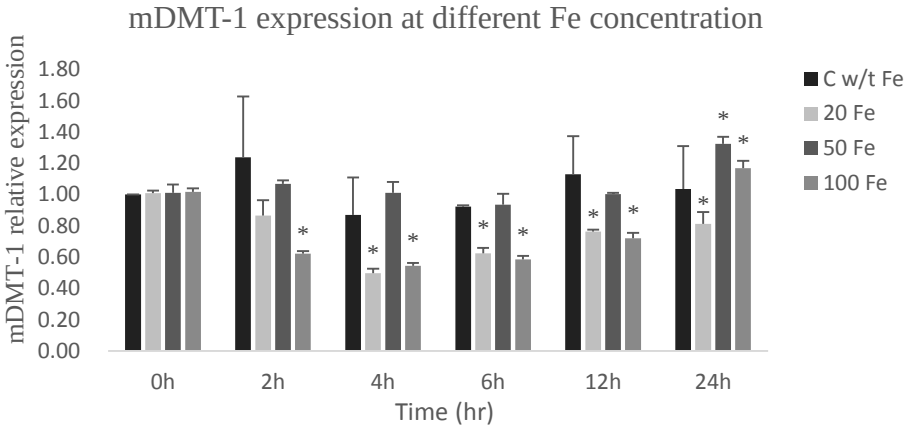
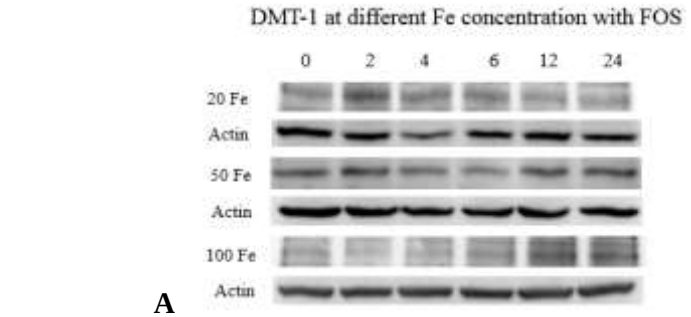


C

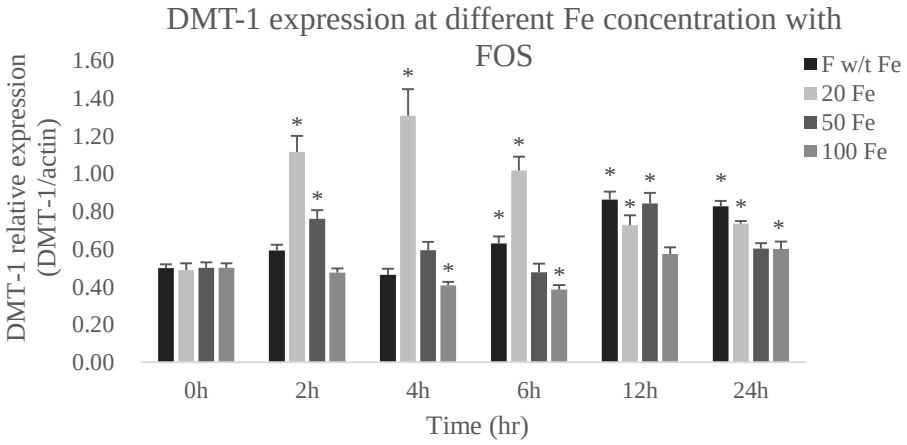
Fig. 3.22 Expression of DMT-1 in cells treated with 20 μM, 50 μM and 100 μM Fe at different time points: A. western bands; B. DMT-1 protein relative expression; C. DMT-1 mRNA relative expression (* Means significantly different compared to the 0 hr time point, $p<0.05$)

When cells were supplemented with FOS together with iron, the effect of 20 μM Fe on the protein expression increased after 2 hr and remained elevated after 24 hr (Fig. 3.23). However, Fig. 3.24 suggested that at 2 hr, cells with just 20 μM Fe and no FOS might actually produce a higher level of DMT-1 protein. FOS with 50 μM Fe led to increases in DMT-1 protein expression at 2 and 12 hr, but there was no clear trend observed (Fig. 3.23). For FOS with 100 μM Fe, there were differences at 4 and 6 hr (decrease), and at 24 hr (increase), but the effects were mild.

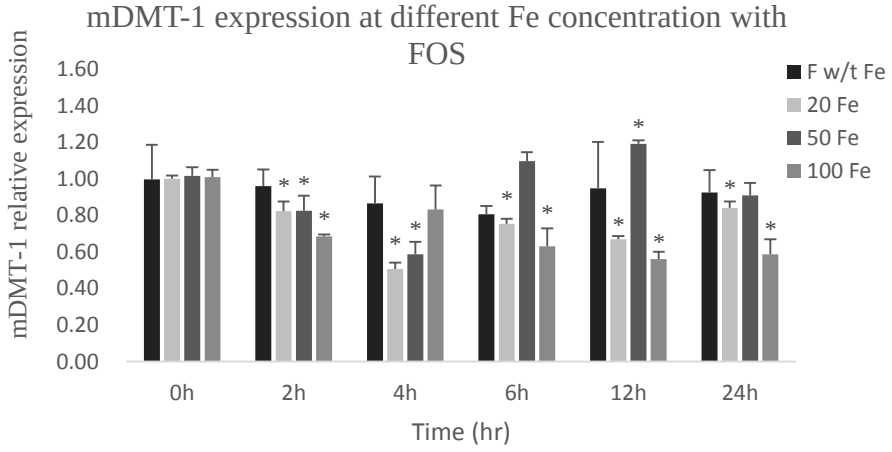
For DMT-1 mRNA expression, the FOS treatments with iron mostly led to comparable or lower expression in cells. The only exception was the increase in DMT-1 mRNA at 12 hr in cells treated with FOS with 50 μM Fe (Fig. 3.23). Overall, the pattern observed in DMT-1 expression in cells treated with FOS with iron was similar to Dcytb expression, which showed an enhanced protein level in cells with FOS at low iron concentration (20 μM), but no or relatively mild effects on mRNA.



A



B



C

Fig. 3.23 Expression of DMT-1 in cells treated with FOS together with 20 μM, 50 μM and 100 μM Fe at different time points: A. western bands; B. DMT-1 protein relative expression; C. DMT-1 mRNA relative expression (* Means significantly different compared to the 0 hr time point, $p<0.05$)

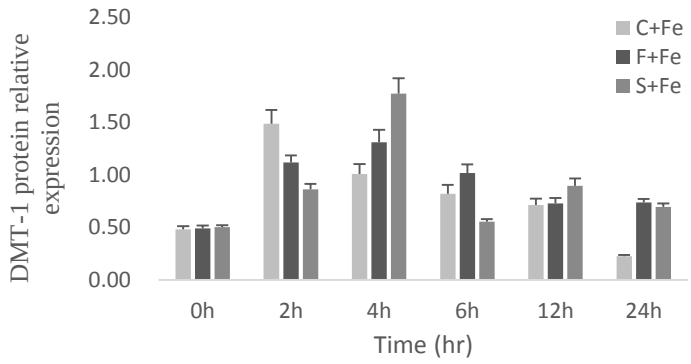
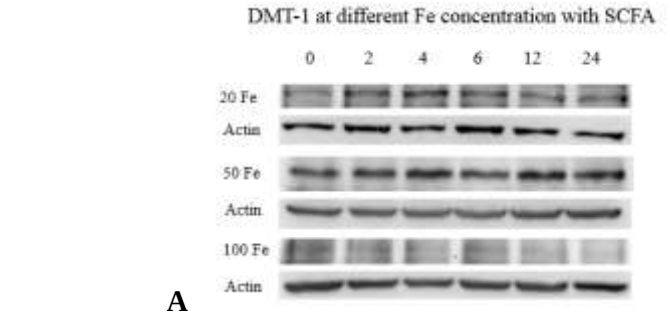


Fig. 3.24 Protein expression of DMT-1 in cells treated with FOS or SCFA together with 20 μM Fe (C = Control; F = FOS; S = SCFA)

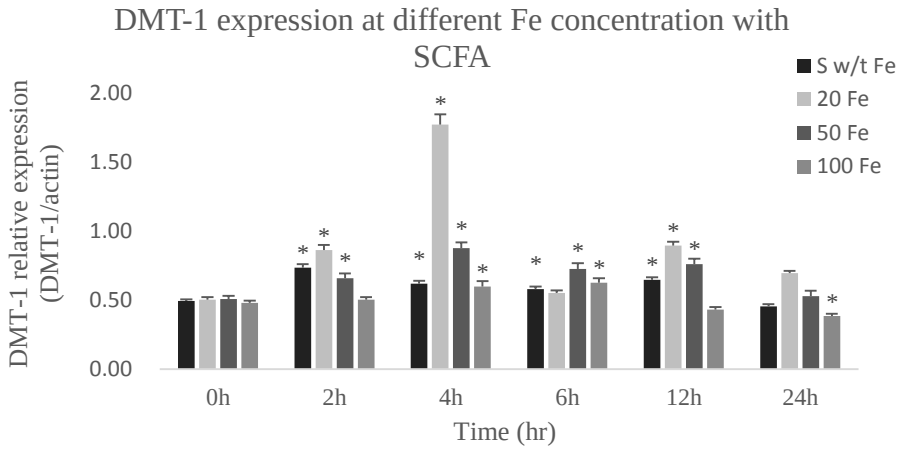
As for cells treated with SCFA together with iron, DMT-1 protein expression increased at different time points between 2 to 12 hr for different concentrations of iron, with the strongest increase observed at 4 hr with 20 μM Fe (Fig. 3.25).

At the mRNA level, SCFA without iron had the most obvious enhancing effect on DMT-1 expression. And the enhancement in mRNA level was much higher than SCFA with any iron concentrations at all time points, except at 4 hr. SCFA with 20 μM Fe had no positive influence on DMT-1 mRNA level. At 50 μM Fe, DMT-1 mRNA was first up-regulated after 2 hr, but then down-regulated after 4 , 12 and 24 hr. As for high concentration of iron (100 μM), DMT-1 mRNA level significantly increased after 4, 6 and 24 hr.

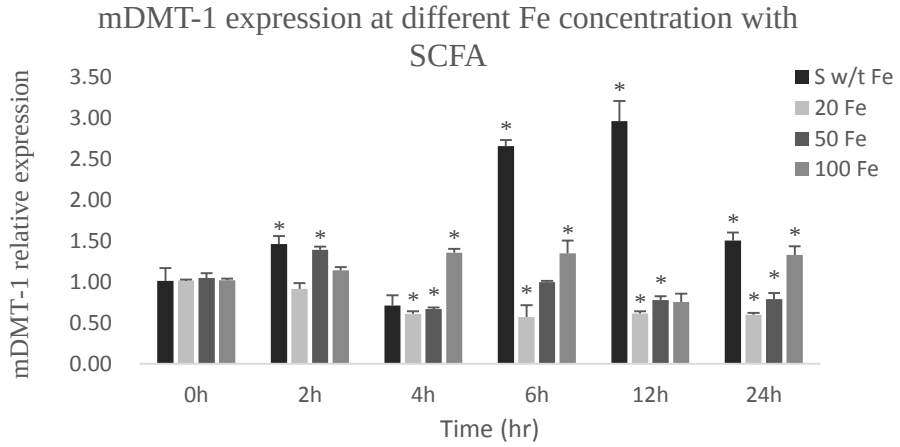
These results showed that SCFA with or without iron could increase DMT-1 protein expression, and effects were more obvious at 20 μM Fe. At the mRNA level, SCFA without iron induced the most enhancing effects. These observations were consistent with those made for Dcytb expression (Section 3.3.2.1).



A



B



C

Fig. 3.25 Expression of DMT-1 in cells treated with SCFA together with 20 μM, 50 μM and 100 μM Fe at different time points: A. western bands; B. DMT-1 protein relative expression; C. DMT-1 mRNA relative expression (* Means significantly different compared to the 0 hr time point, $p<0.05$)

3.5 Discussion

Iron (Fe) is an essential micronutrient for human, and iron deficiency is a leading nutritional deficiency affecting people all over the world. The poor bioavailability of iron in the diet is thought to be one of the key reasons for iron deficiency. Besides, a lack of iron absorption promoters in diet is also considered a limiting factor of dietary iron availability (Yeung et al. 2003). In recent years, non-digestible prebiotics, such as FOS, have been attracting more attention for their potential abilities to enhance mineral absorption (including iron), as well as their functional properties for developing healthy foods (Laparra et al. 2008). In our previous animal studies, FOS had been shown to mildly improve the iron status of anemic rats (Zhang et al. 2017). However, the mechanisms involved are not well elucidated. It is important to understand the interactions between FOS and iron absorption, and the underlying mechanisms so as to effectively utilize FOS as a strategy to combat iron deficiency.

Iron absorption is not only regulated systemically by a hormone peptide called hepcidin produced in liver (Nemeth and Ganz, 2009; Knutson, 2010), but also regulated at the site of uptake in the intestinal mucosa by cellular iron levels and hypoxia (Fuqua et al. 2012). At the cellular level, the importance of Dcytb and DMT-1 is evident as iron uptake across the apical membrane of the enterocyte has been shown to be critical in determining the rate of absorption (O'Riordan et al. 1995; O'Riordan et al. 1997).

Our results showed that there were no significant differences in the controls at different time points either in protein or mRNA expression for both iron absorption genes Dcytb and DMT-1. The expression levels of Dcytb and DMT-1 were relatively stable within the timeframe (0 – 24 hr) of our experiments when no treatments other than regular medium were given to the cells. It was important to verify that the Caco-2 cells provided a stable research model so that any changes in expression levels in subsequent experiments could be attributed to the treatments with FOS, SCFA, or iron.

As seen from the results of cells treated with iron, iron obviously influenced the protein as well as mRNA expression of Dcytb and DMT-1. The extents of the influence were different in protein expression and mRNA expression, and were dependent on iron concentration. For Dcytb protein expression, the low iron concentration (20 μM) induced a quick (i.e., beginning at 2 hr) and strong enhancing effect but the effect diminished with increasing iron concentration, suggesting that Dcytb would be up-regulated when the right amount of iron was present. This is consistent with the results of Mckie et al. (2001) which showed that the reductase activity of Dcytb might be stimulated when proper amount of iron was present, and the expression of Dcytb increased in iron deficient situation.

For DMT-1 protein expression, 20 and 50 μM Fe had similar positive effects, and 20 μM Fe had a stronger effect at early time points (e.g., 2 hr). Zoller et al. (2002) showed that DMT-1 protein expression increased when Caco-2 cells were treated with desferrioxamine, a reagent that binds free iron, indicating that low iron

level could promote the protein expression of DMT- 1. However, they also demonstrated that cells treated with 50 μM FeCl3 for 24 – 60 hr decreased DMT-1 protein and mRNA expression, but DMT-1 protein was still able to be detected by western blot. Our experiments used a 0 – 24 hr timeframe, and it is possible that 50 μM Fe could increase DMT-1 protein expression in early hours, but the effect could diminish with time beyond 24 hr. The high level iron (100 μM) showed no positive effects on either protein or mRNA, and at some time points, even a decrease in expression (e.g., 4 hr on both Dcytb and DMT-1 expression). Johnson et al. (2005) reported that DMT-1 was relocalized away from the brush border membrane of intestinal epithelial cells due to a high level of iron (100 μM) and this process could occur within a few hours (4hr). Other studies also showed that high iron treatment (200 μmol/L) down-regulated DMT-1 in cells (Martini et al., 2001) and down-regulated Dcytb and DMT-1 expression in rats (Frazer et al., 2003) by10 mg oral iron administration after 3 hr.

The trends observed in this study in protein expression of Dcytb and DMT-1 were not mirrored in mRNA expression at low iron levels. Both Dcytb and DMT-1 were reported to be regulated by HIF (hypoxia-inducible factor) responsive elements (HREs) in transcription promoters (Mastrogiannaki et al. 2009; Shah et al. 2009), When HIF, which is a part of transcription factor complex, binds to the HREs containing promoters, transcription will be induced. HIF level increases when iron level decreases, and therefore promotes the transcription of Dcytb and DMT-1 in order to increase iron absorption (Mastrogiannaki et al. 2009; Shah et al.

2009). Transcription and translation of DMT-1 are also affected by the iron responsive element (IRE) located in the 3' UTR region. In case of iron deficiency, iron regulatory proteins (IRP1 or IRP2) could bind to the IRE and stabilized the DMT-1 mRNA by increasing its half-life and thus increase protein translation (Brie et al. 2012). However, when cellular iron levels are too low, IRP1 would bind to iron instead of binding to mRNA and IRP2 would degrade (Brie et al. 2012). The 20 μM Fe level used in our experiments might be too low to maintain the IRPs and mRNA could not be stabilized and thus resulted in a decrease. The down regulation of DMT-1 mRNA at 20 μM Fe was consistent with the study of Tallkvist et al. (2000).

Moreover, the differences between protein and mRNA expression of Dcytb and DMT-1 may just simply illustrate that regulation of iron absorption involves post-transcriptional regulation and other mechanisms. Studies in the literature showed that rapid regulation of DMT-1 protein was not always accompanied by a corresponding mRNA expression during iron uptake by Caco-2 cells (Johnson et al. 2005; Zoller et al. 2002).

When cells were supplemented with FOS only, there were no significant differences in Dcytb protein and mRNA expression within 24 hr, nor there were any significant changes in DMT-1 mRNA expression, suggesting that unfermented FOS had no influence on Dcytb expression or DMT-1 mRNA expression. FOS is an oligosaccharide with 2-10 repeating units, and without any decomposition process, could not affect the gene expression at the mRNA level directly. However,

there was a significant increase in DMT-1 protein expression after 6 hr. It was reported that another oligosaccharide, chitosan, can enhance drugs transport by affecting the permeability of Caco-2 monolayers, and lowering the cell's pH (Artursson et al. 1994). Presumably, FOS might have a similar influence on the pH condition or permeability of cells, and thus potentially changing the expression of DMT-1 protein. DMT-1 may also be more sensitive than Dcytb to changes in the pH as low pH is required for efficient divalent cation transport with DMT-1 (Gunshin et al. 1997).

When FOS and iron were exposed to Caco-2 cells together, FOS could promote Dcytb and DMT-1 protein expression at 20 μM Fe condition after 4hr (Fig 3.16 and Fig 3.23). When iron concentration went up to 50 μM, the protein expression of Dcytb and DMT-1 relatively decreased when compared to the iron-only condition. Similar observations were made at 100 μM iron level, with the exception at 4 hr for Dcytb and 12 hr for DMT-1, when protein expression was enhanced. The overall results suggested that FOS could help to increase protein expression at low iron level but decrease protein expression at higher iron levels. Possible explanations may be that the cells were more sensitive to the iron conditions rather than the unfermented FOS, or due to the mineral binding effect of FOS (Laparra et al. 2008).

At the transcription level, 20 μM Fe with FOS showed no obvious effects on Dcytb and DMT-1 mRNA expression when compared to that without FOS. Laparra et al. (2008) suggested that inulin did not impair iron uptake as mRNA expression

of Dcytb and DMT-1 was similar with or without inulin. The inulin they used was Synergy which was the 1:1 mix of long and short chain FOS. The concentration of iron used in their study was 12 μM which was close to our low iron level (20 μM). Furthermore, in their study, Dcytb mRNA expression in cultures exposed to different iron solutions, with or without added inulin, all presented a similar down-regulation. Their results were consistent ours in that Dcytb mRNA expression at 20 μM iron level with or without FOS also showed similar decreases. However, at 50 μM Fe, mRNA level of Dcytb decreased while DMT-1 was not affected. The decreased expression of Dcytb did not lead to a decrease in DMT-1 might imply that there are other mechanism or reductase for ferric iron reduction at the site of uptake. Zhang et al. (2006) described a candidate, which was a new isoform of cytochrome b561, for ferric reductase activity that could also assist DMT-1 transfer. At 100 μM Fe, Dcytb mRNA was up-regulated by FOS, while DMT-1 mRNA was down-regulated. The effects of FOS at different iron concentrations on mRNA varied and the trends were not obvious.

The positive effects of FOS on iron absorption usually were due to its hydrolyzed product-SCFAs (Trinidad et al., 1993; Yasuda et al., 2006). According to our results, SCFA treatment could enhance the Dcytb and DMT-1 expression on both protein and mRNA levels with or without iron. Protein expression of Dcytb and DMT-1 significantly increased after 2 hr until 12 hr and mRNA expression of Dcytb and DMT-1 was significantly up-regulated from 2 – 24 hr. When cells were supplemented with SCFA together with 20 μM Fe, the strongest enhancing effect

on Dcytb and DMT-1 protein expression could be seen, but SCFA without iron induced the highest expression at the transcriptional level. Salovaara et al. (2002) reported that propionic and acetic acids increased ferrous iron uptake in Caco-2 cells, but no effects on ferric iron under 10 μM Fe. Their results suggested that propionic and acetic acids promoted the gene expression related to ferrous iron transport. Based on the results in our rat studies (Chapter 2), fermentation products of FOS include a mix of acetic, propionic and butyric acids. In general, SCFAs promoted protein expression that would enhance iron absorption.

At the mRNA level, SCFA treatment could increase Dcytb expression at all iron concentrations, but the enhancing effects diminished with higher iron concentrations, except a notable increase at the 24 hr time point at 100 μM Fe. As for DMT-1, SCFA had no obvious effects at 20 and 50 μM Fe, but increased the expression at 100 μM Fe. Other studies showed that DMT-1 mRNA was down-regulated by increasing cellular iron status (Han et al. 1999; Tandy et al. 1999). Our DMT-1 results did not show the same pattern, and further investigations are needed to explain this discrepancy.

Overall, SCFA, and to a lesser extent FOS, had a promotional effect on protein expression at 20 μM iron level, but the effect of SCFA on protein expression was much more pronounced than FOS after 4 hr (Fig 3.16 and Fig 3.23). Data from our Caco-2 cell experiments supported the conclusion from our rat studies that diets supplemented with FOS improved the iron status of anemic rats. Fermentation products of FOS in the colon of rats, namely SCFAs, were the primary contributor

of the enhancing effect, and this is consistent with what has been reported in the literature (Trinidad et al., 1993; Yasuda et al., 2006). In other words, fermentation of dietary FOS by intestinal microflora is necessary for FOS to have an enhancing effect on iron status.

3.6 Conclusions

Caco-2 cells provide a research model that is suitable for gene expression studies related to iron absorption. Expression of Dcytb and DMT-1 at the mRNA level and at the protein level in Caco-2 cells could be measured and quantified with RT-PCR and western blot techniques. Caco-2 cells also respond adequately to iron treatments, allowing researchers to study the effects of different dietary factors. However, it should be noted that based on our results, a measurable increase in Dcytb or DMT-1 protein in Caco-2 cells was not always accompanied by a corresponding measurable increase in mRNA, suggesting that other post-transcriptional factors might be involved. Also at high iron level, expression of Dcytb or DMT-1 was mostly down-regulated.

In terms of the potential effects of FOS on iron absorption, while the effects of FOS on Dcytb and DMT-1 at different iron concentrations varied and might not show a consistent enhancement, effects of its fermentation products, SCFAs, were much more pronounced. Our results suggest that any effects of dietary FOS on improving iron status would require fermentation by the intestinal microflora. Further studies on other prebiotics (e.g., GOS and lactulose) as well as different combinations of SCFAs are warranted.

Chapter 4 Summary

Iron deficiency is one of the most significant public health concerns in the world, affecting 1/3 of the world population, especially in women and children. A more efficient absorption of iron from normal daily diets rich in iron absorption enhancers could be considered a strategy for preventing iron deficiency.

All prebiotics used in the current project showed no adverse effects on iron status. FOS common in plant foods and GOS derived from lactose were proven to be positive in improving the iron status of anemic rats with low iron intake. Our results from the animal studies also indicated that the structure and chain length of prebiotics could influence their effects on iron absorption. Short chain oligosaccharides were potentially more effective than longer chain oligosaccharides in improving iron status. Product developers could consider incorporating FOS and GOS not only in foods and beverages to enhance the consumer appeal, but also in functional food products targeting populations at a higher risk of iron-deficiency anemia.

Gene expression studies using the Caco-2 cell culture model provided supportive results that were consistent with the conclusions drawn from the animal studies. Expression of Dcytb and DMT-1 proteins increased at low iron condition, especially when supplemented with SCFAs. And at high iron level, expression of Dcytb or DMT-1 was mostly down-regulated. Our results also suggested that the effects of dietary FOS on improving iron status would require fermentation by the intestinal microflora. While the effects of FOS on Dcytb and DMT-1 expression at

different iron concentrations varied, the effects of its fermentation products, SCFAs, were much more pronounced. It should be noted that any increases in Dcytb or DMT-1 proteins in Caco-2 cells were not always accompanied by the corresponding increases in mRNA levels, suggesting that other mechanisms might be involved in the post-transcriptional regulation of these genes.

This research supports the previous notion that the Caco-2 cell line is a suitable research model for gene expression studies related to iron absorption. Expression of Dcytb and DMT-1 could be measured in Caco-2 cells semi-quantitatively at both mRNA level and protein level by RT-PCR and western blot techniques. Caco-2 cells also responded adequately to iron treatments, allowing the effects of different dietary factors to be investigated.

In addition to Dcytb and DMT-1, other gene products involved in iron absorption such as ferritin and ferroportin can be included in future studies. The effects of SCFAs on iron absorption also warrant further investigations. Experiments could be designed to determine the combinations of SCFAs produced upon fermentation of different prebiotics by probiotic bacteria, and how different combinations SCFAs affect the regulatory mechanism of iron absorption.

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