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Supplementation of Fructooligosaccharide Mildly Improves the Iron Status of Anemic Rats Fed a Low-Iron Diet

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Abstract

Also known as a prebiotic, fructooligosaccharide (FOS) resists digestion by gastric acid and pancreatic enzymes *in vivo*, but is preferentially fermented by beneficial intestinal bacteria once it reaches the colon. While some studies suggest that FOS and its fermentation products may influence the iron absorption process, the effects of prolonged FOS supplementation on iron status remain unclear. The objective of this study was therefore to determine the enhancing effects of FOS supplementation on the iron status of anemic rats. Male Sprague-Dawley rats receiving a low-iron diet (12 μg/g) for 14 days showed significantly lower hemoglobin concentration, as well as lower tissue non-heme iron levels than rats receiving a regular diet (45 μg/g), confirming iron-deficiency anemia. On the first day of the feeding trial, two groups of anemic rats (n = 6) were fed the same low-iron diet with or without FOS supplementation, while two other groups of anemic rats were switched to the regular diet with or without FOS supplementation to allow recovery. FOS was provided to the rats by dissolving in water at 5% (w/v). Anemic rats fed the low-iron diet showed a mild increase ($p < 0.05$) in hemoglobin level after 21 days of FOS supplementation when compared to rats without FOS. For anemic rats switched to the regular diet, hemoglobin level returned to normal after 14 days and FOS supplementation showed no additional effects. Our results suggest that FOS supplementation has a mild enhancing effect on the iron status of anemic subjects on a low-iron diet.

Keywords

Iron, Fructooligosaccharide, Anemia, Prebiotic

1. Introduction

Fructooligosaccharide (FOS), and several other non-digestible but soluble oligosaccharides such as lactulose and galactooligosaccharide, are known as prebiotics. Prebiotics selectively stimulate the growth and activity of specific species of bacteria in the colon, usually *Bifidobacteria* and *Lactobacilli*, with benefits to health [1]. Langlands *et al.* [2] 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. 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 [3], modulation of the immune system [4], and regulation of neuroendocrine stress response [5]. FOS occurs naturally in a wide variety of plants such as artichokes, asparagus and chicory roots [6], and it is commercially available as an ingredient for foods and beverages.

Iron deficiency is one of the most prevalent nutrient deficiencies in the world, affecting mostly young children and women of child-bearing age in developing countries [7] [8]. Prebiotics may also have an enhancing effect on iron absorption and several biologically plausible mechanisms have been suggested [9]. While a stable isotope study by Patterson *et al.* [10] with anemic piglets surgically fitted with cecal cannulas showed that FOS had no enhancing effect on iron absorption in the colon, Tako *et al.* [11] 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 [12] 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.* [13] showed that children aged 1 - 3 receiving milk fortified with a combination of *B. Lactis HN*109 (1.9×10^7 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.* [14] 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.* [15] 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.

The objectives of this study were to determine the effects 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.

2. Materials and Methods

2.1. Animal Protocol

The experimental protocol was 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).

2.2. Chemicals

Chemicals used in tissue non-heme iron analysis were obtained from Sigma-Aldrich Inc. China (Shanghai, China) or Fisher Scientific (Guangzhou, China) unless stated otherwise. FOS (BENEO Orafti® P95 Oligofructose) was obtained from the Food Ingredients Division of Guangzhou DPO Co. Ltd (Guangzhou, China). Water used in experiments was purified with the Milli-Q® Reference Ultrapure water purification system (EMD Millipore, Billerica, MA).

2.3. Rats

Weanling two-week-old male Sprague-Dawley rats with a mean body weight of <40 g 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.

2.4. 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).

2.5. Experimental Design

A total of 48 rats were used in the experimental design (Table 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, one 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.

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

Table 1. Experimental 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 this study.

supplementation (Table 1). 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).

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 measurement. At the end of the feeding trial, all rats were sacrificed. Liver, spleen, kidney and heart samples were harvested, and weighed portions were used for non-heme iron analysis.

2.6. 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. 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) × concentration of standard (g/dL).

2.7. 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˚C until analysis. Tissue non-heme iron levels were determined by the colorimetric method described by Rebouche *et al.* [16]. 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).

2.8. Statistical Analysis

All statistical analyses were done using Minitab® Release 16 (Minitab Inc., State College, PA). Differences in hemoglobin concentration and tissue non-heme iron in rats with or without FOS were analyzed by the Student's t-test. A p-value of <0.05 was considered significant.

3. Results and Discussion

3.1. Acclimation and Iron-Deficiency Anemia

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 the present study, 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. As noted in the experimental design (Table 1), rats received either a regular diet (45 μg Fe/g diet) to achieve normal iron status, or a low-iron diet (12 μg Fe/g diet) to induce iron-deficiency anemia. After 14 days of acclimation, rats receiving the low-iron diet showed lower hemoglobin concentration, as well as lower tissue non-heme iron levels in liver, spleen, kidney and heart, when compared to normal rats (Table 2). In a recent study on iron-copper interactions, Ha *et al.* [17] 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. Our results showed that rats receiving the low-iron diet for 14 days reached a hemoglobin concentration of 5.0 g/dL, and also had a significantly lower body weight ($p < 0.05$) than the normal rats (87.0 g vs 101.9 g). These observations are consistent with the report by Ha *et al.* [17], confirming that the rats receiving the low-iron diet achieved iron-deficiency anemia after the acclimation period.

3.2. Weight Gain and Water Intake

All rats gained weight in a linear fashion during the feeding trial (Figure 1). Daily observations revealed no signs of diarrhea in rats. There were no significant

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

	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)	17.7 ± 0.9[a]	8.6 ± 0.5[b]
Kidney non-heme iron (μg/g)	45.9 ± 6.1[a]	15. 9 ± 0.9[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 ($p < 0.05$).

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, Day 28, Day 35 or Day 42). As in the study by Ha *et al.* [17], anemic rats remaining on the low-iron diet during the feeding trial gained weight at a slower rate (smaller weekly percent increases) compared to normal rats on the regular diet. However, anemic rats switched to the regular diet were able to gain weight essentially at the same rate as normal rats with or without FOS (**Figure 1**). Overall, FOS did not affect weight gain in anemic or normal rats.

There were no significant differences in daily water intake during the feeding trial between rats receiving the same diet with or without FOS (**Table 3**). The only exception was the water intake during the first week of feeding trial (Day 15 - 21) by the pair of anemic rat groups switched to the regular diet (**Table 3**). There was no clear explanation for this anomaly, as there were no differences in body weight between the pair, and subsequent daily water intakes were not different either. 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

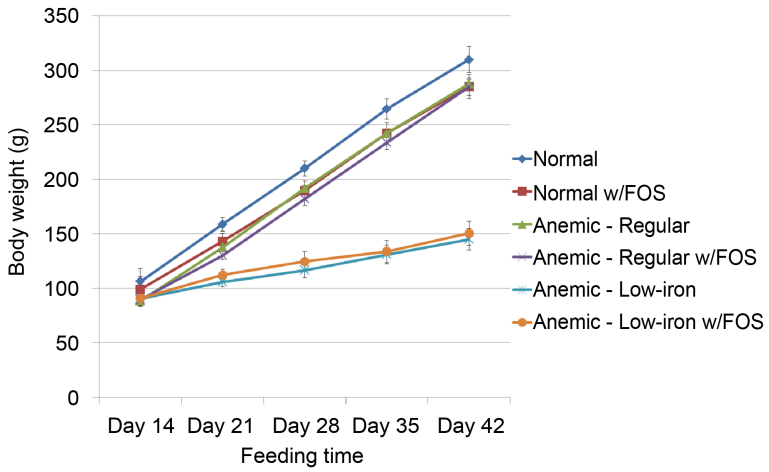

Figure 1. Weekly changes in body weight (Mean ± SEM) in normal and anemic rats with or without FOS. Error bars indicate standard error of mean (n = 6). No significant differences were observed in body weight between each pair of rat groups with or without FOS at any time points.

Table 3. Rat daily water intake (mL, Mean ± SEM) during the feeding trial.

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

*Daily water intake was significantly lower with FOS ($p < 0.05$).

normal rats (Figure 1), water intake remained relatively low throughout the feeding trial (Table 3). 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 [18]. Nevertheless, the underlying physiological basis for the relatively lower water intake warrants further investigations.

From Table 3, 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 [19]. 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 *et al.* [20] 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 [21]. With an assumed body weight of 70 kg for an average American adult, an acceptable FOS intake would be equal to only 0.43 g/kg body weight.

3.3. Effects of FOS on Iron Status and Recovery from Anemia

At the onset of the feeding trial (Day 14), hemoglobin concentrations of anemic rat groups ranged from 5.0 - 5.6 g/dL (Figure 2). Anemic rats remaining on the low-iron diet continued to show low hemoglobin concentrations throughout the feeding trial. However, after 3 weeks (Day 35), there was a mild increase ($p < 0.05$) in hemoglobin concentration for anemic rats on the low-iron diet with FOS supplementation compared to the ones without (Figure 2). When the rats were sacrificed at the end of the trial, anemic rats on the low-iron diet with FOS supplementation also showed a significantly higher liver non-heme iron level (Figure 3). There were, nevertheless, no differences in tissue non-heme iron levels

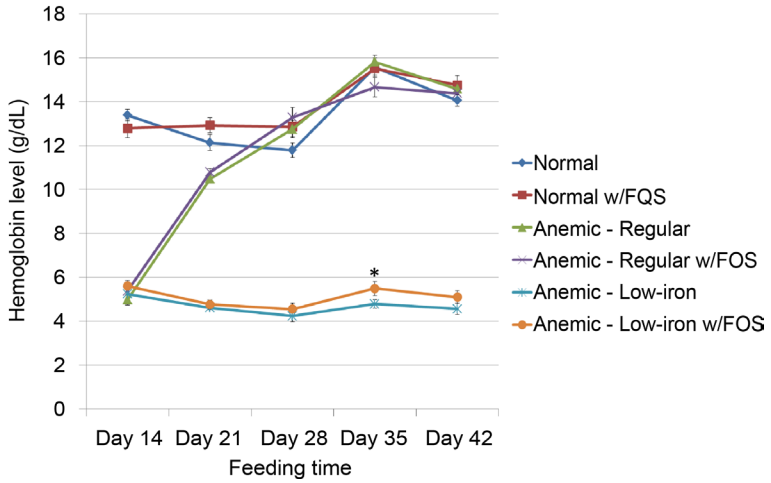

Figure 2. Weekly changes in hemoglobin concentration (Mean ± SEM) in normal and anemic rats with or without FOS. Error bars indicate standard error of mean (n = 6). *Hemoglobin concentration was significantly higher with FOS ($p < 0.05$).

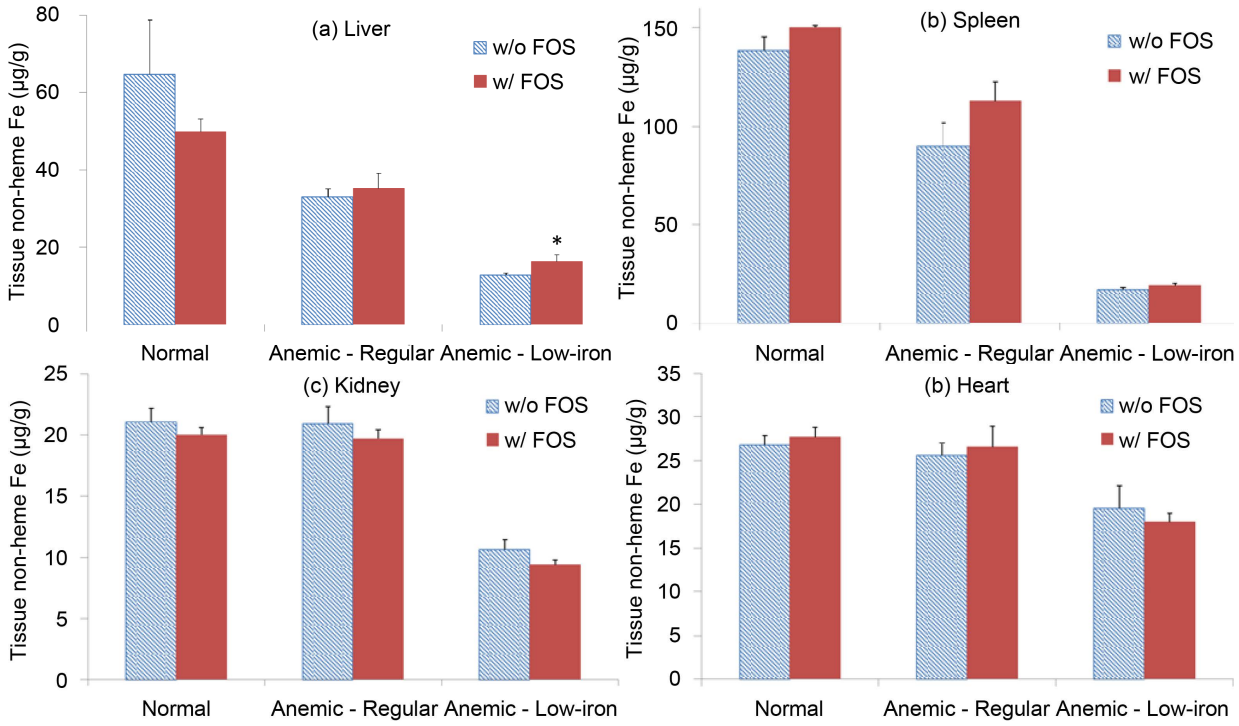

Figure 3. Rat tissue non-heme iron levels (μg/g) after the feeding trial (Day 42) in (a) liver; (b) spleen; (c) kidney; and (d) heart. Error bars indicate standard error of mean (n = 6). *Only liver non-heme iron level of anemic rats fed a low-iron diet was significantly higher with FOS ($p < 0.05$).

in spleen, kidney, or heart. 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 [22] [23]. Our results showed 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 [24]. 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 [9]. 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 [25] [26]. 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 [27], Sakai *et al.* [28] 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 at the onset of the feeding trial were comparable to normal rats after only 2 weeks

(Figure 2). During these 2 weeks of recovery, FOS supplementation did not provide any additional boost, *i.e.*, no significant differences with or without FOS, on Day 21 or Day 28. Tissue non-heme iron levels (liver, spleen, kidney and heart) were not different either at the end of the trial (Figure 3). For normal rats with or without FOS, there were 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 negatively affect iron status.

Dietary factors such as ascorbic acid and meat enhance, whereas milk and phytate inhibit iron absorption [29]. As an ingredient, FOS is soluble and commercially available for various product applications. Incorporation of FOS into foods and beverages may be considered one of the dietary strategies to improve the iron status of population groups suffering from a high risk of iron-deficiency anemia (*i.e.*, young children and women of childbearing age). It should be noted that retention of FOS in food products is heavily dependent on the pH and the time-temperature combinations of thermal treatments used in food processing [30]. Nevertheless, Vega and Zuniga-Hansen [31] showed that FOS retention rate could be 70% - 95% with high-temperature short-time (HTST) processing even in acidic beverages such as orange and tomato juices, suggesting that FOS could survive typical pasteurization processes and be available to the consumer.

4. Conclusion

FOS supplementation mildly improves the iron status of anemic rats with a low iron intake. While FOS may not have any additional effects on recovery from anemia in rats already with adequate iron intake, FOS supplementation does not show any adverse effects on iron status. Product developers should consider incorporating FOS 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.

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References

[1] Cummings, J.H., Macfarlane, G.T. and Englyst, H.N. (2001) Prebiotic Digestion and Fermentation. *The American Journal of Clinical Nutrition*, **73**, 415s-420s.

[2] Langlands, S.J., Hopkins, M.J., Coleman, N. and Cummings, J.H. (2004) Prebiotic Carbohydrates Modify the Mucosa Associated Microflora of the Human Large Bowel. *Gut*, **53**, 1610-1616. https://doi.org/10.1136/gut.2003.037580

[3] Spiegel, J.E., Rose, R., Karabell, P., Frankos, V.H. and Schmitt, D.F. (1994) Safety and Benefits of Fructooligosaccharides as Food Ingredients. *Food Technology*, **48**, 85-89.

[4] Jeurink, P.V., van Esch, B.C., Rijnierse, A., Garssen, J. and Knippels, L.M. (2013) Mechanisms Underlying Immune Effects of Dietary Oligosaccharides. *The American Journal of Clinical Nutrition*, **98**, 572S-577S. https://doi.org/10.3945/ajcn.112.038596

[5] Schmidt, K., Cowen, P.J., Harmer, C.J., Tzortzis, G., Errington, S. and Burnet, P.W. (2015) Prebiotic Intake Reduces the Waking Cortisol Response and Alters Emotional Bias in Healthy Volunteers. *Psychopharmacology*, **232**, 1793-1801. https://doi.org/10.1007/s00213-014-3810-0

[6] Gorski, D. (1997) Ingredient Forecast Product Development for World Markets. *Dairy Foods*, **98**, 60-62.

[7] Lynch, S.R. (2011) Why Nutritional Iron Deficiency Persists as a Worldwide Problem. *The Journal of Nutrition*, **141**, 763S-768S. https://doi.org/10.3945/jn.110.130609

[8] Camaschella, C. (2015) Iron-Deficiency Anemia. *The New England Journal of Medicine*, **372**, 1832-1843. https://doi.org/10.1056/NEJMra1401038

[9] Yeung, C.K., Glahn, R.E., Welch, R.M. and Miller, D.D. (2005) Prebiotics and Iron Bioavailability—Is There a Connection? *Journal of Food Science*, **70**, R88-R92. https://doi.org/10.1111/j.1365-2621.2005.tb09984.x

[10] Patterson, J.K., Rutzke, M.A., Fubini, S.L., Glahn, R.P., Welch, R.M., Lei, X. and Miller, D.D. (2009) Dietary Inulin Supplementation Does Not Promote Colonic Iron Absorption in a Porcine Model. *Journal of Agricultural and Food Chemistry*, **57**, 5250-5256. https://doi.org/10.1021/jf900698x

[11] Tako, E., Glahn, R.P., Welch, R.M., Lei, X., Yasuda, K. and Miller, D.D. (2008) Dietary Inulin Affects the Expression of Intestinal Enterocyte Iron Transporters, Receptors and Storage Protein and Alters the Microbiota in the Pig Intestine. *British Journal of Nutrition*, **99**, 472-480. https://doi.org/10.1017/S0007114507825128

[12] Samolińska, W. and Grela, E.R. (2017) Comparative Effects of Inulin with Different Polymerization Degrees on Growth Performance, Blood Trace Minerals, and Erythrocyte Indices in Growing-Finishing Pigs. *Biological Trace Element Research*, **176**, 130-142.

[13] Sazawal, S., Dhingra, U., Hiremath, G., Sarkar, A., Dhingra, P., Dutta, A., Menon, V.P. and Black, R.E. (2010) Effects of *Bifidobacterium lactis* HN019 and Prebiotic Oligosaccharide Added to Milk on Iron Status, Anemia, and Growth among Children 1 to 4 Years Old. *Journal of Pediatric Gastroenterology and Nutrition*, **51**, 341-346.

[14] Coudray, C., Bellanger, J., Castiglia-Delav, C., Remesy, C., Vermorel, M. and Rayssignuier, Y. (1997) Effect of Soluble or Partly Soluble Dietary Fibres Supplementation on Absorption and Balance of Calcium, Magnesium, Iron and Zinc in Healthy Young Men. *European Journal of Clinical Nutrition*, **51**, 375-380. https://doi.org/10.1038/sj.ejcn.1600417

[15] Van den Heuvel, E.G., Schaafsma, G., Muys, T. and van Dokkum, W. (1998) Nondigestible Oligosaccharides Do Not Interfere with Calcium and Nonheme-Iron Absorption in Young, Healthy Men. *The American Journal of Clinical Nutrition*, **67**, 445-451.

[16] Rebouche, C.J., Wilcox, C.L. and Widness, J.A. (2004) Microanalysis of Non-Heme Iron in Animal Tissues. *Journal of Biochemical and Biophysical Methods*, **58**, 239-251. https://doi.org/10.1016/j.jbbm.2003.11.003

[17] Ha, J.H., Doguer, C., Wang, X., Flores, S.R. and Collins, J.F. (2016) High-Iron Consumption Impairs Growth and Causes Copper-Deficiency Anemia in Weanling Sprague-Dawley Rats. *PLoS ONE*, **11**, e0161033. https://doi.org/10.1371/journal.pone.0161033

[18] Felt, B.T., Beard, J.L., Schallert, T., Shao, J., Aldridge, J.W., Connor, J.R., Georgieff, M.K. and Lozoff, B. (2006) Persistent Neurochemical and Behavioral Abnormalities in Adulthood Despite Early Iron Supplementation for Perinatal Iron-Deficiency Anemia in Rats. *Behavioural Brain Research*, **171**, 261-270. https://doi.org/10.1016/j.bbr.2006.04.001

[19] National research Council. (1995) Nutrient Requirements of Laboratory Animals: 1995. National Academies Press, Washington DC.

[20] Moshfegh, A.J., Friday, J.E., Goldman, J.P. and Ahuja, J.K.C. (1999) Presence of Inulin and Oligofructose in the Diets of Americans. *The Journal of Nutrition*, **129**, 1407S-1411S.

[21] Coussement, P.A.A. (1999) Inulin and Oligofructose: Safe Intakes and Legal Status. *The Journal of Nutrition*, **129**, 1412S-1417S.

[22] Mejia, L.A., Hodges, R.E. and Rucker, R.B. (1979) Role of Vitamin A in the Absorption, Retention and Distribution of Iron in the Rat. *The Journal of Nutrition*, **109**, 129-137.

[23] Andrews, N.C. (1999) Disorders of Iron Metabolism. *The New England Journal of Medicine*, **341**, 1986-1995. https://doi.org/10.1056/NEJM199912233412607

[24] Stewart, M.L., Timm, D.A. and Slavin, J.L. (2008) Fructooligosaccharides Exhibit More Rapid Fermentation than Long-Chain Inulin in an *in Vitro* Fermentation System. *Nutrition Research*, **28**, 329-334. https://doi.org/10.1016/j.nutres.2008.02.014

[25] Shiga, K., Nishimukai, M., Tomita, F. and Hara, H. (2006) Ingestion of Difructose Anhydride III, a Non-Digestible Disaccharide, Prevents Gastrectomy-Induced Iron Malabsorption and Anemia in Rats. *Nutrition*, **22**, 786-793. https://doi.org/10.1016/j.nut.2005.12.013

[26] Suzuki, T., Nishimukai, M., Shinoki, A., Taguchi, H., Fukiya, S., Yokota, A., Saburi, W., Yamamoto, T., Hara, H. and Matsui, H. (2010) Ingestion of Epilactose, a Non-Digestible Disaccharide, Improves Postgastrectomy Osteopenia and Anemia in Rats through the Promotion of Intestinal Calcium and Iron Absorption. *Journal of Agricultural and Food Chemistry*, **58**, 10787-10792. https://doi.org/10.1021/jf102563y

[27] Bougle, D., Vaghefi-Vaezzadeh, N., Roland, N., Bouvard, G., Arhan, P., Bureau, F., Neuville, D. and Maubois, J.L. (2002) Influence of Short-Chain Fatty Acids on Iron Absorption by Proximal Colon. *Scandinavian Journal of Gastroenterology*, **37**, 1008-1011. https://doi.org/10.1080/003655202320378176

[28] Sakai, K., Ohta, A., Shiga, K., Takasaki, M., Tokunaga, T. and Hara, H. (2000) The Cecum and Dietary Short-Chain Fructooligosaccharides Are Involved in Preventing Postgastrectomy Anemia in Rats. *The Journal of Nutrition*, **130**, 1608-1612.

[29] Collings, R., Harvey, L.J., Hooper, L., Hurst, R., Brown, T.J., Ansett, J., King, M. and Fairweather-Tait, S.J. (2013) The Absorption of Iron from Whole Diets: A Systematic Review. *The American Journal of Clinical Nutrition*, **98**, 65-81. https://doi.org/10.3945/ajcn.112.050609

[30] Duar, R.M., Ang, P.T., Hoffman, M., Wehling, R., Hutkins, R. and Schlegel, V. (2015) Processing Effects on four Prebiotic Carbohydrates Supplemented in an Extruded Cereal and a Low pH Drink. *Cogent Food & Agriculture*, **1**, Article ID: 1013782. https://doi.org/10.1080/23311932.2015.1013782

[31] Vega, R. and Zuniga-Hansen, M.E. (2015) The Effect of Processing Conditions on the Stability of Fructooligosaccharides in Acidic Food Products. *Food Chemistry*, **173**, 784-789. https://doi.org/10.1016/j.foodchem.2014.10.119