Synergistic effect of galacto oligosaccharides and iron fortificants on serum iron, ferritin, transferrin and total iron binding capacity levels in anemic rats

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Abstract: Iron deficiency anemia is one of the leading public health issues being faced by the global population currently. The present research was an attempt to determine the synergistic effect of Galacto Oligosaccharides and iron fortificants on serum iron, serum ferritin, serum transferrin and total iron binding capacity in anemic rats. To serve the purpose, eight different types of fortified feed were prepared with varying concentrations of Iron Fortificants (NaFeEDTA and $FeSO_4$) while the varying dosage of galacto oligosaccharides was dissolved separately in water to be fed to anemic rats. Afterwards, animal trials were conducted for twelve weeks to determine the efficacy of Galacto Oligosaccharides & iron fortificants based feed against the aforementioned parameters. The results of the study suggested that both serum iron and serum ferritin levels were significantly improved when anemic rats were fed with iron and Galacto Oligosaccharides fortified feed. It was also observed that the levels of serum transferrin and total iron binding capacity steadily decreased over the study duration. It can be concluded that Galacto Oligosaccharides helped enhance the absorption of iron in anemic rats, reflected by increase in serum iron and serum ferritin levels and decrease in serum transferrin and total iron binding capacity.

Keywords: Galacto-oligosaccharides, prebiotics, NaFeEDTA, $FeSO_4$, iron fortificants, serum iron, serum ferritin.

INTRODUCTION

Micronutrient is a broad terminology which is used to refer to minerals and vitamins provided by the diet, for the sustenance of all normal molecular and cellular processes, taking place in a living organism (la Cruz-Góngora *et al.,* 2012). Micronutrients are needed by the human body in very minute amounts, but their deficiencies may have long term health consequences which can also lead to death if untreated. MNDs (Micronutrient deficiencies) are common all across the globe and affect approximately 2 billion people, which refer to approximately 25% of the globe's population (Addis Alene and Mohamed Dohe, 2014).

Most frequently occurring micronutrient deficiencies include those of Vitamin A, Iron, Folate, Zinc and Iodine. Vitamin D deficiency and Selenium deficiency are also common in certain areas of the world. MNDs generally exist as component of a vicious malnutrition cycle and may well be associated with protein energy malnutrition (Bailey *et al.,* 2015).

The word anemia comes from Greek language, which literally means "lack of blood" and it arises when there is a decrease in either the number of red blood cells or total hemoglobin content of the body. Iron deficiency anemia is that particular form of anemia, which occurs due to insufficient iron, needed to synthesize red blood cells. All across the globe, the most common cause of anemia is deficiency of iron (Johnson-Wimbley and Graham, 2011).

A prebiotic is defined as a non viable food substance component (insoluble fiber), which exhibits selective fermentation and moves to the colon. The term prebiotic comes from Greek language, which means "for life" and was initially used to refer to substances secreted by a microorganism, which had the ability to stimulate the growth of another microorganism. The term was therefore used in contrast to the term "antibiotic" (Schrezenmeir and de Vrese, 2001). Prebiotics are not digested in the small intestine, rather they escape to the colon, where they function as a growth substrate for the intestinal bacteria. Most common examples of prebiotics include Inulin, Galacto oligosaccharides, Fructo oligosaccharides, Xylo oligosaccharides and Mannan oligosaccharides (Simmering and Blaut, 2001).

Intake of prebiotics has been associated with the prevention of the colon cancer, assistance in the absorption of minerals such as magnesium, calcium and iron, reduction of cholesterol levels (thereby reducing the risk of cardiovascular diseases), reduction of the severity of IBD (Inflammatory Bowel Disease) and weight loss (Slavin, 2013).

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Various studies have suggested that prebiotics specifically Galacto-Oligosaccharides and Inulin exhibit iron absorption increasing capabilities. This particular characteristic exhibited by both of these prebiotics can be exploited to treat iron deficiency anemia which is one of the biggest public health issues worldwide (Sundberg, 2011).

In a study conducted in the year 2001, it was shown that as much as 10% of dietary oligofructose helped in the stimulation of iron retention in growing rat models. It was also observed that oligofructose helped in the recovery from anemia, induced by diet (Scholz-Ahrens *et al.,* 2001).

One other study concluded that anemia could be prevented in rat models by feeding them oligofructose, which increased the iron absorption in the gut. Similarly, several other studies have concluded that the concentrations of Hemoglobin, Hematocrit and other Serum Iron biomarkers were increased in animal models, after they were fed with diet rich in prebiotics (Bougle *et al.,* 2002).

In another study, the researchers made two experimental groups of Sprague Dawley rats and gave Xylo-Oligosaccharides rich diet to one group while control diet to the second group. At the end of trials, it was observed that the group which received diet rich in Xylo-Oligosaccharides had absorbed iron significantly better, compared to the control group. Moreover, values such as Hematocrit, Hemoglobin, Total Iron Binding Capacity and Transferrin Saturation were also elevated in group fed with Xylo-Oligosaccharides rich diet (Kobayashi *et al.,* 2011).

MATERIALS AND METHODS

Raw materials and chemicals

Chemicals and raw materials required for the research were bought from well reputed companies such as Fluka and Sigma Aldrich.

Production and optimization of GOS

The enzyme β-galactosidase was purchased and transgalactosylation of lactose (600mM) prepared in 50 mM NaPP buffer, pH 6.5 at 37°C using 300 rpm was done. The reaction was ceased by heating the mixture at a temperature of 90^{o}C for 5 minutes and samples were stored at -20^{o}C. Analysis of lactose, glucose and galactose was done using Megazyme assay kits (Ireland) within the transgalactosylated mixture by employing the standard protocol available in the manual. Galacto oligosaccharides were then calculated using the formula; GOS = Initial concentration of lactose - (Glucose + Galactose + Untransgalactosylated Lactose) The final mixture of GOS thus obtained was eventually subjected to TLC (Thin Layer Chromatography) to ensure that the resultant GOS had high purity (Maawia *et al*. 2016).

Experimental animals

For the present research study, 70 young (6 to 8 weeks old) female Sprague Dawley rats were obtained from NIH (National Institute of Health), Islamabad.

Galacto oligosaccharides & iron fortificants based feed

Galacto Oligosaccharides & Iron Fortificants based fortified feed was manually prepared for the purpose of this study.

Treatment plan

For the purpose of research, eight different types of fortified feed with varying concentrations of Iron Fortificants were prepared. Varying dosage of Galacto oligosaccharides was dissolved in water while iron fortificants were added to the basal diet, whose composition was based on standards.

Galacto oligosaccharides & iron fortificant dose galacto oligosaccharide dose

The recommended dose of prebiotics for human consumption is 6 to 8 grams per day. The prebiotic dosage for the present research was calculated using the human equivalent dose (HED) equation after weighing the actual rats (Reagan-Shaw *et al*. 2008).

HED (mg/kg) = Animal Dose in mg/kg × (Animal Weight in kgs) / (Human Weight in kgs)$^{0.33}$

Iron fortificant dose

Doses of iron fortificants used in the trials were 10 ppm and 20 ppm for NaFeEDTA and 15 ppm and 30 ppm for $FeSO_4$.

Feed design

Control feed design

D_+ was the positive control, that is, it had neither added iron fortificant nor the galacto oligosaccharides and was fed to the group of healthy rats. D_- was the negative control, that is, it contained no added fortificant or galacto oligosaccharides and was fed to the anemic rats (table 1).

Treatments feed design

Galacto-Oligosaccharides and NaFeEDTA Based Feed Design

Four types of galacto-oligosaccharides and sodium ferric ethylenediaminetetraacetate based feed were prepared (table 2).

Table 1: Treatment plan (Control Feed Design)

Group	Diet plan
D_+	Positive control (Basal Diet/Water/No added fortificant/no added galacto oligosaccharides) - Fed to healthy rats
D_-	Negative Control (Basal Diet/Water/No Added Fortificant/No Added Galacto Oligosaccharides) - Fed to Anemic Rats

Table 2: Treatment plan (Galacto-oligosaccharides and NaFeEDTA based feed design)

Group	Diet plan	Human equivalent dose (HED) - For GOS
D_1	Basal Diet + 722 mg/kg GOS + 10 ppm NaFeEDTA	100 mg/kg = 6 grams
D_2	Basal Diet + 722 mg/kg GOS + 20 ppm NaFeEDTA	100 mg/kg = 6 grams
D_3	Basal Diet + 963 mg/kg GOS + 10 ppm NaFeEDTA	133 mg/kg = 8 grams
D_4	Basal Diet + 963 mg/kg GOS + 20 ppm NaFeEDTA	133 mg/kg = 8 grams

Table 3: Treatment Plan (Galacto-oligosaccharides and $FeSO_4$ based feed design)

Group	Diet plan	Human equivalent dose (HED) - For GOS
D_5	Basal Diet + 722 mg/kg GOS + 15 ppm $FeSO_4$	100 mg/kg = 6 grams
D_6	Basal Diet + 722 mg/kg GOS + 30 ppm $FeSO_4$	100 mg/kg = 6 grams
D_7	Basal Diet + 963 mg/kg GOS + 15 ppm $FeSO_4$	133 mg/kg = 8 grams
D_8	Basal Diet + 963 mg/kg GOS + 30 ppm $FeSO_4$	133 mg/kg = 8 grams

Table 4: Mean squares for serum iron, serum ferritin, transferrin and total iron binding capacity in anemic female rats fed with iron fortificants and GOS Fortified Feed

SOV	df	Serum iron	Serum Ferritin	Serum transferrin	Total iron binding capacity
Groups	9	696.02*	1949.80*	138.60*	2771.90*
Study Intervals	3	662.57*	9692.70*	189.10*	5538.30*
Groups x Study Intervals	27	127.40*	353.60*	130.40*	299.00*
Error	240	4.54	1.0	128.70	20.10
Total	279				

* = Significant (P-value < 0.05)

Table 5: Serum iron levels (µg/dL) among anemic female rats from baseline up to 90 days

Treatments/ Groups	Days				Means
	0	30	60	90	
D_+	84.98 ± 1.19	83.80 ± 1.83	83.10 ± 1.55	84.18 ± 1.57	84.01 ± 0.78
D_-	77.70 ± 2.77	71.49 ± 2.51	67.98 ± 2.26	64.42 ± 1.39	70.39 ± 5.66
D_1	79.26 ± 2.60	83.62 ± 2.70	84.19 ± 1.77	87.57 ± 1.54	83.66 ± 3.41
D_2	78.05 ± 2.66	82.42 ± 2.51	85.02 ± 1.94	88.53 ± 1.72	83.50 ± 4.42
D_3	77.47 ± 1.91	81.79 ± 2.13	83.94 ± 1.59	87.33 ± 1.83	82.63 ± 4.13
D_4	77.97 ± 1.46	86.42 ± 1.44	92.83 ±1.45	95.84 ± 1.88	88.26 ± 7.91
D_5	77.82 ± 1.56	82.96 ± 1.45	90.98 ± 1.28	94.50 ± 1.22	86.56 ± 7.57
D_6	78.52 ± 1.81	83.64 ± 1.64	90.03 ± 1.30	93.57 ± 1.37	86.44 ± 6.69
D_7	77.45 ± 1.22	82.62 ± 1.31	78.97 ± 2.57	82.65 ± 3.24	80.42 ± 2.63
D_8	78.01 ± 1.81	83.25 ± 1.83	79.19 ± 4.85	82.34 ± 4.44	80.69 ± 2.50
Means	78.72 ± 2.26	82.20 ± 3.96	83.62 ± 7.22	86.09 ± 9.01	

Where, D_+ = Unfortified feed (healthy control), D_- = Unfortified feed (anemic control), D_1 = Basal Diet + 722 mg/kg GOS + 10 ppm NaFeEDTA, D_2 = Basal Diet + 722 mg/kg GOS + 20 ppm NaFeEDTA, D_3 = Basal Diet + 963 mg/kg GOS + 10 ppm NaFeEDTA, D_4 = Basal Diet + 963 mg/kg GOS + 20 ppm NaFeEDTA, D_5 = Basal Diet + 722 mg/kg GOS + 15 ppm $FeSO_4$, D_6 = Basal Diet + 722 mg/kg GOS + 30 ppm $FeSO_4$, D_7 = Basal Diet + 963mg/kg GOS + 15 ppm $FeSO_4$, D_8 = Basal Diet + 963mg/kg GOS + 30 ppm $FeSO_4$

Table 6: Serum ferritin levels (ng/mL) among anemic female rats from baseline up to 90 days

Treatments/ Groups	Days				Means
	0	30	60	90	
D_+	23.47 ± 1.62	22.86 ± 1.10	22.86 ± 1.42	23.68 ± 1.35	23.21± 0.42
D_-	19.51 ± 0.18	17.60 ± 0.36	15.89 ± 1.23	15.03 ± 1.02	17.00 ± 1.97
D_1	18.35 ± 0.56	43.19 ± 0.95	45.82 ± 1.04	53.03 ± 1.24	40.09±15.08
D_2	18.06 ± 0.84	37.91 ± 2.89	45.44 ± 0.73	52.72 ± 0.94	38.53±14.93
D_3	18.30 ± 0.53	36.67 ± 0.82	45.60 ± 0.86	52.84 ± 1.11	38.35±14.91
D_4	18.73 ± 0.52	40.65 ± 0.77	53.92 ± 1.09	65.83 ± 1.43	44.78±20.18
D_5	18.48 ± 0.47	36.65 ± 0.65	44.74 ± 0.79	49.74 ± 0.73	37.40±13.72
D_6	18.29 ± 0.48	36.34 ± 0.41	44.55 ± 0.60	49.53 ± 0.50	37.17±13.72
D_7	18.11 ± 0.89	36.27 ± 0.74	44.44 ± 0.81	49.41 ± 0.76	37.05±13.74
D_8	18.08 ± 0.83	36.21 ± 0.93	44.32 ± 1.05	49.43 ± 1.18	37.01±13.74
Means	18.93 ± 1.65	34.43 ± 7.92	40.75±11.74	46.12±15.06	

Where, D_+ = Unfortified feed (healthy control), D_- = Unfortified feed (anemic control), D_1 = Basal Diet + 722 mg/kg GOS + 10 ppm NaFeEDTA, D_2 = Basal Diet + 722 mg/kg GOS + 20 ppm NaFeEDTA, D_3 = Basal Diet + 963 mg/kg GOS + 10 ppm NaFeEDTA, D_4 = Basal Diet + 963 mg/kg GOS + 20 ppm NaFeEDTA, D_5 = Basal Diet + 722 mg/kg GOS + 15 ppm $FeSO_4$, D_6 = Basal Diet + 722 mg/kg GOS + 30 ppm $FeSO_4$, D_7 = Basal Diet + 963 mg/kg GOS + 15 ppm $FeSO_4$, D_8 = Basal Diet + 963 mg/kg GOS + 30 ppm $FeSO_4$

Table 7: Transferrin levels (mg/dL) among anemic female rats from baseline up to 90 days

Treatments/ Groups	Days				Means
	0	30	60	90	
D_+	19.52 ± 0.23	19.53 ± 0.20	19.52 ± 0.11	19.43 ± 0.16	19.50 ± 0.05
D_-	20.93 ± 0.08	21.00 ± 0.09	21.10 ± 0.10	21.21 ± 0.06	21.06 ± 0.12
D_1	20.97 ± 0.18	20.76 ± 0.22	20.44 ± 0.17	20.02 ± 0.17	20.54 ± 0.41
D_2	20.89 ± 0.19	20.60 ± 0.07	20.36 ± 0.21	19.92 ± 0.23	20.44 ± 0.41
D_3	20.95 ± 0.15	20.53 ± 0.08	20.42 ± 0.18	19.98 ± 0.21	20.47 ± 0.40
D_4	20.98 ± 0.17	20.26 ± 0.07	19.87 ± 0.21	18.83 ± 0.24	19.98 ± 0.90
D_5	21.02 ± 0.21	20.91 ± 0.09	20.58 ± 0.19	20.02 ± 0.19	20.63 ± 0.45
D_6	21.04 ± 0.21	20.55 ± 0.06	20.59 ± 0.26	20.03 ± 0.26	20.55 ± 0.41
D_7	21.00 ± 0.23	20.65 ± 0.07	20.53 ± 0.31	19.97 ± 0.31	20.53 ± 0.43
D_8	20.94 ± 0.21	20.56 ± 0.04	20.50 ± 0.23	19.94 ± 0.25	20.48 ± 0.41
Means	20.82 ± 0.46	20.53 ± 0.41	20.39 ± 0.43	19.93 ± 0.59	

Where, D_+ = Unfortified feed (healthy control), D_- = Unfortified feed (anemic control), D_1 = Basal Diet + 722mg/kg GOS + 10 ppm NaFeEDTA, D_2 = Basal Diet + 722mg/kg GOS + 20 ppm NaFeEDTA, D_3 = Basal Diet + 963mg/kg GOS + 10ppm NaFeEDTA, D_4 = Basal Diet + 963mg/kg GOS + 20 ppm NaFeEDTA, D_5 = Basal Diet + 722mg/kg GOS + 15 ppm $FeSO_4$, D_6 = Basal Diet + 722mg/kg GOS + 30ppm $FeSO_4$, D_7 = Basal Diet + 963mg/kg GOS + 15 ppm $FeSO_4$, D_8 = Basal Diet + 963mg/kg GOS + 30ppm $FeSO_4$

Galacto-oligosaccharides and feso$_4$ based feed design

Four types of Galacto-Oligosaccharides and Ferrous Sulphate based feed were prepared (table 3).

Efficacy trials

In order to determine the efficacy of pre-biotics & iron fortificants based premixes, bio evaluation trials on experimental rats were conducted. The rats (n=70) were randomly divided into control groups (positive and negative) and treatment groups (total 10 groups), 7 rats in each group, based on the oral diet provision of varying concentrations of galacto oligosaccharides and Iron Fortificants (Sodium Ferric Ethylenediaminetetraacetate and Ferrous Sulphate), as described in table 2 and 3 above.

For the purpose of acclimatization of rats, they were provided with a standard diet for 1 week prior to beginning the experiment. After that, anemia was initially induced among the rats by orally feeding them with iron binders such as triapine and tachpyridine and baseline values were attained. Following that, rats were orally fed pre-biotics and iron fortificants based fortified feed daily for 3 months. Mean body weight of each group of rats was determined at the start of the experiment as well as at the end of every week, so as to adjust the dosage of Galacto Oligosaccharides accordingly. Stainless steel cages were used for housing of rats and a maintenance temperature of 23±2ºC and relative humidity of 55±5% was ensured. Moreover, during the experimental trials, 12 hour light-dark cycle was also maintained.

Table 8: Total iron binding capacity levels (µg/dL) among anemic female rats from baseline up to 90 days

Treatments/ Groups	Days				Means
	0	30	60	90	
D_+	468.48 ± 5.44	468.73 ± 4.75	468.47 ± 2.71	466.41 ± 3.76	468.02±1.08
D_-	502.37 ± 1.96	504.11 ± 2.24	506.48 ± 2.39	509.04 ± 1.48	505.50±2.90
D_1	503.17 ± 4.37	498.20 ± 5.29	490.50 ± 4.09	480.36 ± 4.08	493.05±9.94
D_2	501.25 ± 4.53	494.31 ± 1.63	488.62 ± 5.06	478.19 ± 5.62	490.59±9.75
D_3	502.20 ± 3.56	492.60 ± 1.95	490.13 ± 4.22	479.58 ± 5.10	491.12±9.29
D_4	503.45 ± 4.02	486.18 ± 1.77	476.76 ± 5.06	451.98 ± 5.77	479.59±21.47
D_5	504.56 ± 5.05	501.80 ± 2.08	493.80 ± 4.54	480.40 ± 4.54	495.14±10.83
D_6	504.88 ± 5.15	493.31 ± 1.43	494.04 ± 6.35	480.73 ± 6.21	493.24±9.88
D_7	504.08 ± 5.45	495.60 ± 1.59	492.80 ± 7.37	479.34 ± 7.52	492.95±10.27
D_8	502.66 ± 4.95	493.40 ± 0.93	492.04 ± 5.57	478.44 ± 5.92	491.63±9.98
Means	499.71±11.03	492.82±9.84	489.36±10.28	478.44±14.13	

Where, D_+ = Unfortified feed (healthy control), D_- = Unfortified feed (anemic control), D_1 = Basal Diet + 722 mg/kg GOS + 10 ppm NaFeEDTA, D_2 = Basal Diet + 722 mg/kg GOS + 20 ppm NaFeEDTA, D_3 = Basal Diet + 963 mg/kg GOS + 10 ppm NaFeEDTA, D_4 = Basal Diet + 963 mg/kg GOS + 20 ppm NaFeEDTA, D_5 = Basal Diet + 722 mg/kg GOS + 15 ppm $FeSO_4$, D_6 = Basal Diet + 722 mg/kg GOS + 30 ppm $FeSO_4$, D_7 = Basal Diet + 963 mg/kg GOS + 15 ppm $FeSO_4$, D_8 = Basal Diet + 963 mg/kg GOS + 30 ppm $FeSO_4$

Up to three months, blood samples were collected from overnight fasted rats on monthly basis. In order to draw the blood from rats, tail vein was identified and cleaned using 70% alcohol after which the rats were restrained. Lateral tail vein was the site used to draw blood using 21-gauge needle. After the sample was taken, blood flow was stopped by exerting pressure using sterile gauze so as to achieve the homeostasis. At every withdrawal, it was ensured that no more than 1% of respective rat's body weight was removed for sampling purpose. For terminal blood withdrawal, full general anesthesia was given to the rats and blood was withdrawn using the technique of cardiac puncture. Following the withdrawal, the rats were euthanized using sodium pentobarbital of 100mg/kg (Kumar *et al*. 2017).

Analytical procedures

The collected blood samples from all the groups were subjected to analysis using their respective protocols for Serum Iron (Wojciak *et al.,* 2013), Serum Ferritin (Kazuaki *et al.,* 2011) and Serum Transferrin (Al-Buhairan and Oluboyede, 2000). Total Iron Binding Capacity on the other hand was calculated using the formula; TIBC = Transferrin x 24

STATISTICAL ANALYSIS

In order to determine the level of significance, statistical analysis of the obtained data was done for the purpose of which, SPSS version 23.0 was used. Factorial design was employed during the experiment and differences were considered significant at P - value <0.05 (Steel and Torrie, 1997).

Ethical considerations

The approval of the study was taken from the ERC (Ethical Review Committee) of the University of Veterinary and Animal Sciences, Lahore (No. DR/996) on September 25, 2018.

RESULTS

In our study, it was observed that mean squares for all the four variables under consideration (Serum Iron, Serum Ferritin, Transferrin & Total Iron Binding Capacity) in anemic female Sprague Dawley rats were significantly different with regards to the effect of groups, study intervals as well as their interaction (table 4).

Serum iron

It can be observed from table 5 that maximum value for Serum Iron was recorded in group D_4 (Basal Diet + 963 mg/kg GOS+20 ppm NaFeEDTA) which was 88.26±7.91µg/dL, followed by group D_5 whereby the value was observed to be 86.56±7.57µg/dL. This was followed by groups D_6 and D_1 where the values were recorded to be 86.44±6.69µg/dL and 83.66±3.41µg/dL, respectively. Lowest serum iron levels were observed in group D_- with a value of 70.39±5.66 µg/dL (table 5).

Serum ferritin

As per the means regarding serum ferritin levels, it can be observed that the maximum values were recorded in groups fed with Galacto Oligosaccharides and NaFeEDTA. Amongst these groups, group D_4 had the highest mean value of 44.78±20.18ng/mL, followed by group D_1, D_2 and D_3 with values of 40.09±15.08ng/mL, 38.53±14.93ng/mL and 38.35±14.91ng/mL, respectively (table 6).

Serum transferrin

It can be observed from table 7 that the maximum value of serum transferrin was 21.06±0.12mg/dL which was

observed in group D_- (negative control). Among the treatment groups, maximum values were observed in groups D_5, D_6, D_1 and D_7, which were 20.63±0.45mg/dL, 20.55±0.41mg/dL, 20.54±0.41mg/dL and 20.53±0.43 mg/dL, respectively. Minimum values for serum transferrin were observed in groups D_4, D_2, D_3 and D_8 which were 19.98±0.90mg/dL, 20.44±0.41mg/dL, 20.47±0.40 mg/dL and 20.48±0.41mg/dL, respectively.

Total iron binding capacity

Means regarding total iron binding capacity depict that the maximum value of 505.50±2.90µg/dL for this particular parameter was observed in group D_- (negative control). Among the treatment groups, the highest value was recorded in groups D_5, D_6, D_1 and D_7 with values of 495.14±10.83µg/dL, 493.24±9.88µg/dL, 493.05±9.94 µg/dL and 492.95±10.27µg/dL, respectively. Least values of total iron binding capacity were observed in groups D_4, D_2, D_3 and D_8, having values of 479.59±21.47µg/dL, 490.59±9.75µg/dL, 491.12±9.29µg/dL and 491.63±9.98 µg/dL, respectively (table 8).

DISCUSSION

Several research studies have shown that prebiotics help in the absorption of iron in both animal and human models (Wang 2017). In addition, they are also known to exhibit positive effects on other bone related minerals, such as magnesium, calcium and zinc and thereby help to improve the Bone Mineral Content (BMC) (Scholz-Ahrens *et al.* 2007).

Paganini *et al.,* conducted at study, aimed at investigating whether the intake of prebiotics is associated with enhanced iron absorption among infants or not. To serve the purpose, fifty Kenyan infants were chosen and randomly divided into two groups. Group one was fed with fortified maize porridge with MNP (having Ferrous Fumarate and NaFeEDTA along with 7.5 grams Galacto oligosaccharides) daily for a period of 21 days. Group two was given the same maize porridge but without the addition of prebiotic Galacto oligosaccharides. The results of the study showed that the group fed with MNP and Galacto oligosaccharides had enhanced iron absorption by 62%, compared to other group, possibly due to increased absorption of iron by colon (Paganini *et al.* 2017a). The results of this study are in close harmony with our study as we have also observed an increase in iron absorption due to addition of Galacto oligosaccharides in iron fortificants.

Another study was designed to determine the effects of various prebiotics (Inulin, FOS, GOS and Lactulose) on iron status of anemic rats. The results of the study revealed that iron absorption had been significantly enhanced among rat groups fed with either FOS or GOS. It was further seen that SCFAs (short chain fatty acids) concentration were improved among treatment groups owning to the fermentation of prebiotics in the colon, carried out by probiotics. The study concluded that addition of prebiotics was helpful in enhancing absorption of iron among anemic rats (Zhang 2017).

In another attempt, the peers determined the effects of supplementation of FOS in soya drink on absorption of iron in weaning rats. The conclusions of the study suggested that when rats were provided with FOS supplemented soy drink, not only their hemoglobin levels were improved compared to the control group (P-value< 0.05), but also the expression of DMT 1 protein was enhanced. It was therefore suggested that FOS supplementation helped improve iron absorption among weaning rats (Silva *et al.* 2018), which is in close harmony with our study results as well.

The researchers of another study hypothesized that iron bioavailability from young child formulae (YCF) would be enhanced with the addition of Fructo oligosaccharides (FOS) and Galacto oligosaccharides (GOS). The results of the study revealed that those YCF in which highest concentration of FOS and GOS were added, had maximum iron bioavailability. On the other hand, formulae with least amounts of prebiotics FOS and GOS had minimum iron bioavailability. The authors concluded that a direct relationship existed between presence of prebiotics and iron bioavailability from YCF (Christides *et al.* 2018).

In a study, a new Micronutrient Powder (MNP) formula (with the addition of prebiotic Galacto oligosaccharides) was evaluated in treating iron deficiency anemia among under 5 children. Purposely, a four month randomized controlled trial was designed for Kenyan infants whereby they were divided into 3 groups. The control group was fed with MNP without iron, while the first treatment group was given MNP with 2.5mg NaFeEDTA and 2.5 mg $C_4H_2FeO_4$. The second treatment group was given MNP and 7.5g Galacto oligosaccharides. The results of the study showed that anemia was significantly reduced in both the treatment groups (P-value<0.001). It was furthermore observed that addition of Galacto oligosaccharides helped reduce the negative consequences of high iron dose on gut health. The authors concluded that a relatively lower dose of iron contained in MNP coupled with 7.5g of Galacto oligosaccharides was helpful in not only significantly reducing anemia but in also mitigating the adverse effects of iron on gut health (Paganini *et al.* 2017b). Our study also found out that addition of Galacto oligosaccharides significantly reduced anemia and increased iron absorption among anemic subjects.

Effect on serum iron and serum ferritin levels

In our study, across the feed model trials, serum iron levels improved significantly from 78.72±2.26µg/dL to 82.20±3.96µg/dL, 83.62±7.22µg/dL and 86.09±9.01

µg/dL at 0, 30th, 60th and 90th day, respectively. A relevant improvement in serum iron levels was also observed among the rat groups with progression in study intervals. Maximum improvement could be seen in groups D_4, D_5 and D_6 which ranged from 77.97±1.46µg/dL at 0 day to 95.84±1.88µg/dL at 90th day for group D_4, 77.82±1.56 at 0 day to 94.50±1.22 at 90th day for group D_5 and 78.52±1.81 at 0 day to 93.57±1.37 at 90th day, respectively.

In a previous study, researchers induced iron deficiency anemia in female Wistar rats by feeding them low iron diet and divided them into different groups which were fed on NaFeEDTA (@ 6, 12 and 24 ppm) and $FeSO_4$ (@6, 12 and 24 ppm) based fortified diets. When rats were assessed for serum iron and serum ferritin levels, it was observed that the group fed on 24 ppm $FeSO_4$ had maximum serum iron levels of 139.36±1.58µg/dL. On the other hand, the group fed on 24ppm NaFeEDTA had serum iron levels of 134.10±2.73µg/dL. Serum ferritin levels of these two groups however, were not significantly different. The researchers concluded that iron fortification was one of the most effective strategies to address the problem of iron deficiency anemia (Rubi and Rohman, 2015).

For Serum Ferritin levels, with regards to the feed model trials in our study, there was an improvement in serum ferritin levels starting from 18.93±1.65ng/mL at 0 day to 34.43±7.92ng/mL, 40.75±11.74ng/mL and 46.12±15.06 ng/mL at 30th, 60th and 90th day, respectively.

A likely explanation of why the groups fed with Galacto Oligosaccharides and NaFeEDTA performed better compared to groups fed with Galacto Oligosaccharides and $FeSO_4$ could be that ferrous is a more soluble form of iron and is therefore absorbed more easily compared to ferric form which is present in NaFeEDTA.

With the progression in study intervals, a significant improvement in serum ferritin levels could be observed with maximum improvement observed in group D_4, followed by group D_1, D_2 and D_3. As far as the group D_4 was concerned, the improvement in serum ferritin ranged from 18.73±0.52ng/mL at 0 day to 65.83±1.43ng/mL at 90th day. For groups D_1, D_2 and D_3, serum ferritin levels ranged from 18.35±0.56ng/mL to 53.03±1.24ng/mL, 18.06±0.84ng/mL to 52.72±0.94ng/mL and 18.30±0.53 ng/mL to 52.84±1.11ng/mL at 0 and 90th day, respectively.

Earlier in year 2003, a research was conducted in Vietnam whereby an evaluation of efficacy of fish sauce fortified with iron was done as fish sauce is one of the staple foods of the region. It was a randomized controlled trial in which 152 anemic women were served with fish sauce containing 10 mg iron in the form of NaFeEDTA for a period of 6 months. At the time of initiation of study, baseline values of hemoglobin and serum ferritin were obtained and the same data was collected at 90th and 180th day. After a period of 6 months, it was observed that mean value for hemoglobin was 116.30±8.7g/L in the fortified group, compared to control group in which the mean value was recorded to be 107.60±11.0g/L (P-value <0.0001). Similarly, mean value for serum ferritin levels was found out to be 30.90 (95% CI: 23.4, 40.6) µg/L in the fortified group compared to 14.6 (11.3, 19.0) µg/L in the control group. (P-value = 0.0002) (Thuy *et al*. 2003). Our study results are also in accordance with this research study as we have also stated that serum ferritin levels in anemic subjects steadily increased from 0 day to 90th day when anemic subjects were given iron and Inulin fortified diet.

Gera *et al.,* in 2012 have reported results from 60 randomized controlled trials stating that various foods fortified with iron could result in increase in serum ferritin (1.36µg/L 95% CI: 1.23, 1.52; P<0.001) as well as hemoglobin levels (0.42g/dL; 95% CI: 0.28, 0.56; P< 0.001) and also resulted in decreased risk of anemia (RR: 0.59; 95% CI: 0.48, 0.71; P<0.001) and that of iron deficiency (RR: 0.48; 95% CI: 0.38, 0.62; P<0.001) (Kobayashi *et al.*, 2011). Our study results are also of the similar nature as we have also reported an increase in serum ferritin levels of anemic rats fed with iron fortified diets.

Effect on serum transferrin and total iron binding capacity levels

Among rat groups, a steady decrease in serum transferrin was observed while the study intervals were progressed, which indicated that iron stores were being improved among anemic rats. This is because transferrin is known to increase as soon as an individual develops iron deficiency anemia indicating that the body requires more iron (Cable *et al.,* 2016).

Maximum decline in serum transferrin levels was observed in group D_4 ranging from 20.98±0.17mg/dL at baseline to 18.83±0.24 at the termination, which was followed by group D_2 whereby serum transferrin declined from 20.89±0.19mg/dL at 0 day to 19.92±0.23mg/dL at 90th day (table 7).

As the study trials progressed, a steady decline in the levels of total iron binding capacity was observed which referred to the fact that the overall body reserves of iron in anemic rats were being improved. It is a well known fact that in case of iron deficiency anemia, total iron binding capacity levels are increased indicating that iron stores are low. On the other hand, among the healthy individuals or when iron deficiency anemia is being treated, TIBC levels tend to decline indicating a definite improvement in body iron stores (Asif *et al.,* 2016).

In our study, the variations in TIBC levels ranged from 499.71±11.03 µg/dL at baseline to 492.82±9.84 µg/dL, 489.36±10.28 µg/dL and 478.44±14.13 µg/dL at 30th, 60th and 90th days, respectively. Within the groups, a systematic decline in TIBC levels was observed when they were given GOS and iron fortificant based fortified feeds. Maximum decline was recorded in group D_4 (503.45±4.02µg/dL at 0 day to 451.98±5.77µg/dL at 90th day), followed by group D_2 (501.25±4.53µg/dL at 0 day to 478.19±5.62µg/dL at 90th day) and D_3 (502.20±3.56 µg/dL at 0 day to 479.58±5.10µg/dL at 90th day), respectively.

A study was conducted by *Lobo et al.* in 2011 in order to determine the effect of prebiotics on iron bioavailability from $FeSO_4$ and Ferric Pyrophosphate in iron deficient rats. The results of the study revealed that serum iron levels were improved in iron deficient rats along with an increase in caecal fermentation. They also reported an increase in serum ferritin levels of rats along with a steady decline in total iron binding capacity, which indicated that iron reserves in the body were being restored (Lobo *et al.,* 2011).

CONCLUSION

Our findings confirmed that when Galacto Oligosaccharides were added to iron fortificants for the treatment of iron deficiency anemia, serum iron and serum ferritin levels were improved in anemic rats. This indicates that Galacto Oligosaccharides have the potential to enhance the absorption of iron in the body, thereby improving body iron reserves. Given the global magnitude of the problem of iron deficiency anemia, this particular research should further be translated into human subjects to determine if a similar response is generated in human subjects.

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