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Iron and prebiotic fortified flour improves the immune function of iron deficient women of childbearing age

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Abstract: Micronutrient deficiencies (MNDs) are common worldwide, in both developing as well as developed countries. MNDs such as Iron Deficiency not only compromise the nutritional status of individuals but can also put them at an increased risk of developing various other diseases by negatively affecting their immunity. The objective of the current research was to determine the effects of prebiotics and iron fortificants on various immunoglobulins among iron deficient women belonging to childbearing age. To serve the purpose, a total of seventy five iron deficient women were selected and randomly divided into one control and four treatment groups. Accordingly, different types of fortified wheat flour were prepared, based on varying dosage of prebiotics and iron fortificants, to be fed to anemic women on daily basis for three months. Two iron salts (FeSO4 and NaFeEDTA) and two prebiotics (Galacto oligosaccharides and Inulin) were used to fortify wheat flour during the trials. Overnight fasted women were asked to give blood samples on monthly basis, up to three months. Four types of Immunoglobulins including IgA, IgE, IgG and IgM were determined at baseline, 30^{th}, 60^{th} and 90^{th} day of trials using their respective protocols. The results of the study indicated that a statistically significant declining trend for IgA, IgE, IgG and IgM was present among the treatment groups (P-value < 0.05), compared to the control group. The study concluded that provision of iron and prebiotic fortified flour improved the immune function of iron deficient women.

Keywords: Micronutrient deficiencies, iron deficiency anemia, immunoglobulins, antibodies, immunity.

INTRODUCTION

Hidden hunger is a huge global public health issue these days and refers to deficiency of minerals and/or vitamins. The fact is that although minerals and vitamins are required by humans in very small amounts on daily basis, yet their deficiencies can cause grave health consequences, which might also be irreversible in certain cases (Harding *et al.,* 2018). Deficiencies of various micronutrients are common in different regions of the world, depending on the specific etiological factors. However, there are certain micronutrients whose deficiencies are prevalent in almost every part of the world which include Iron, Zinc, Vitamin A, Folate and Iodine (Bailey *et al.,* 2015). Micronutrient deficiencies not only affect the nutritional status of individuals, but can also deteriorate their health status, especially those who are vulnerable (Berti *et al.,* 2014). With regards to developing micronutrient deficiencies, age groups which are most susceptible include under 5 children, women of child bearing age and elderly (Saini *et al.,* 2016).

Anemia is a term which comes from the Greek language and literally means "lack of blood". There are several types of anemia and the most important ones are Iron Deficiency Anemia, Pernicious Anemia and Megaloblastic Anemia. However, the most commonly occurring form of anemia is that of Iron Deficiency Anemia (Lopez *et al.,* 2016).

Clinically, iron deficiency anemia is defined as an insufficient mass of Erythrocytes in blood circulation. In terms of public health, Iron Deficiency Anemia is generally defined as lower levels of hemoglobin in the blood or sometimes as reduced hematocrit levels (Lopez *et al.,* 2016). According to the World Health Organization, no single cut off for Iron Deficiency Anemia can be accepted and it varies according to the age, sex and physiological status of an individual. For example, for non pregnant adult women, hemoglobin levels below 12g/dL categorize them as being anemic. On the other hand, pregnant adult women are deemed to be anemic when their hemoglobin levels fall below 11g/dL (Breymann, 2015).

The etiology of iron deficiency anemia is multi factorial. insufficient oral intake of iron, decreased absorption by the body, enhanced physiological requirements and long term iron loss (due to hemorrhage etc) are among the leading risk factors for iron deficiency anemia (Chen *et al.,* 2013). Iron also interacts with some of the other nutrients such as zinc and calcium as they all compete for absorption in the body. This can also lead to diminished bioavailability of iron in individuals who have intake of

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such food combinations (Skibsted, 2016). In developing countries, the major underlying cause of anemia has been considered to be poverty (Kassebaum, 2016).

The term Dietary Fiber is very commonly used in modern nutrition science and it was first coined in 1953. However, the use of what now constitutes "Dietary Fiber" in the treatment of various ailments has been extremely common for centuries (Slavin, 2013). Dietary fiber is defined as those carbohydrate polymers and oligomers which escape digestion by small intestine and are fermented by large intestine (Verspreet *et al.,* 2016). Research conducted during last few decades has shown that dietary fiber is excellent for human health as it can prevent various chronic diseases such as hypertension, diabetes mellitus, obesity, stroke, and some forms of cancers especially those associated with the gastrointestinal tract. Moreover, it has also been shown to reduce low density lipoprotein cholesterol levels thereby reducing the risk of cardiovascular diseases. Furthermore, prebiotic fiber has now been proven to help in considerable weight loss by giving a feeling of early satiety (Fuller *et al.,* 2016).

A prebiotic is defined as a non viable food substance component (insoluble fiber) (Roberfroid 2007; Sanders *et al.* 2014; Bindels *et al.* 2015; Valcheva and Dieleman 2016), which exhibits selective fermentation and moves to the colon to ferment bacteria, thereby improving the host health (Pandey *et al.* 2015; Hutkins *et al.* 2016).

For foods which fall under the definition of prebiotics, they ought not to be absorbed or hydrolyzed in the upper part of the gastrointestinal tract, should have the ability to exhibit a positive influence on the gut microflora, should pose some beneficial effects to host health and should be a selective substrate for at least some of the colonic bacteria (Markowiak and Slizewska 2017; Wilson and Whelan 2017).

There are several examples of prebiotics but the most common ones include fructo oligosaccharides, galacto oligosaccharides, xylo oligosaccharides, mannan oligosaccharides and inulin (Jacobs, 2017). Foods which are the best sources of prebiotics are jerusalem artichoke, chicory, onion, garlic, leeks, chickpeas, lentils, barley, oats and bran (Florowska *et al.,* 2016).

Most commonly utilized vehicle in the world for the purpose of iron fortification is wheat flour currently (Pachon *et al.* 2015). A systematic review conducted by the researchers suggested that fortification of wheat flour with iron in 20 countries had significantly improved the iron status of populations and also significantly contributed to the decline of prevalence of anemia. Moreover, in 24 countries, iron fortified wheat flour was found to be moderately efficacious in terms of reducing the disease burden of anemia (Sadighi *et al.,* 2015).

Wheat is the staple food of Pakistan and offers more than 50% of the total energy intake (Akhtar *et al.* 2011). Wheat flour fortification program was started in Pakistan back in 2007 at Khyber Pakhtunkhwa (KPK) initially but has now spread to all the provinces of the country. In Pakistan, seven wheat flour fortification campaigns have been launched since 2005, out of which two have been on national level and five on regional level (Ansari *et al.* 2018). Widespread efforts are now being made by the government as well as the private sector to raise awareness among people and also involved all the stakeholders in order to fight the menace of micronutrient deficiencies especially iron deficiency anemia by consumption of iron fortified wheat flour (Akhtar *et al.* 2013).

Immunoglobulins also known as antibodies are glycoprotein in nature, which are produced by WBCs (white blood cells) (Perez *et al.,* 2017). They play an extremely important role in the immunity of an individual, as they have the natural ability to recognize and bind foreign agents in the body (antigens) such as bacteria or viruses and help to destroy them (Berlot *et al.,* 2015).

MATERIALS AND METHODS

Raw materials and chemicals

Wheat flour needed for the research was purchased from the local market while chemicals such as iron fortificants were bought from the famous Sigma Aldrich. Prebiotic Inulin was extracted from Chicory roots while prebiotic galacto oligosaccharides (GOS) were prepared from lactose.

Extraction of Inulin

For the purpose of extraction of Inulin, Chicory roots were taken and washed with water. They were then cut into small pieces and after that, subsequent drying and grinding was done using a laboratory grinder. Extraction of Inulin was done by taking 100 grams of ground sample powder and stirring at 900C for 30 minutes using 0.9g NaCl and 600mL distilled water. Filtration of this stirred powder was done, followed by precipitation with Ethanol at 40C for 24 hours. It was then centrifuged at 300 rpm for 20 minutes after which it was washed thrice with ethanol. Finally, oven drying of the resultant powder was carried out at 400C to obtain the Inulin powder (Bouaziz *et al.*, 2014). Once the extraction process was completed, the inulin powder was subjected to the processes of ultrafiltration and spray drying to obtain the pure inulin powder. An adequate membrane was used which ensured the retaining of high molecular inulin particles within the retenate. The resultant inulin obtained after this process was approximately 98.6% pure. 100 grams of chicory root yielded approximately 4.5 to 5 grams of Inulin (Berghofer *et al.,* 1993).

Table 1: Treatment Plan (Iron Fortificants & Prebiotics Based Diet)

Groups	Diet Plan	Human Equivalent Dose (HED) for Prebiotic
G_0	Control (No prebiotic + 20 ppm NaFeEDTA)	-
G_1	963 mg/kg Inulin + 10 ppm NaFeEDTA	133 mg/kg = 8 grams
G_2	963 mg/kg Inulin + 20 ppm NaFeEDTA	133 mg/kg = 8 grams
G_3	963 mg/kg GOS + 15 ppm $FeSO_4$	133 mg/kg = 8 grams
G_4	963 mg/kg GOS + 30 ppm $FeSO_4$	133 mg/kg = 8 grams

Table 2: Mean Squares regarding Immunoglobulins for Anemic Women fed with Prebiotic and Iron Fortified Diet

Source of Variation	Degrees of Freedom	IgA	IgE	IgG	IgM
Groups	4	2702.49*	3669.87*	32068.20*	42734.40*
Study intervals	3	720.41*	1033.84*	10777.40*	4544.80*
Groups x Study Intervals	12	20.15*	16.53*	2821.20*	1135.70*
Error	280	1.64	3.14	7.20	6.00
Total	299				

*=Significant (P-value <0.05) Where, IgA =Immunoglobulin A, IgE =Immunoglobulin E, IgG =Immunoglobulin G, IgM =Immunoglobulin M

Table 3: Effect of Fortified Diets on Immunoglobulin A (mg/dL) among Anemic Women

Treatments/ Groups	Days				Means ± SD
	0	30	60	90	
G_0	200.91 ± 0.66	201.09 ± 0.98	198.97 ± 1.05	196.23 ± 1.20	199.30 ± 2.26
G_1	214.75 ± 1.39	212.33 ± 1.82	209.24 ± 2.06	205.58 ±2.75	210.47 ± 3.97
G_2	210.56 ± 1.26	207.42 ± 2.34	203.48 ± 4.55	200.37 ±4.67	205.45 ± 4.46
G_3	204.54 ± 1.31	201.29 ± 2.95	198.56 ± 2.99	195.68 ± 3.22	200.01 ± 3.79
G_4	199.97 ± 1.34	194.84 ± 2.56	191.90 ±3.65	186.22 ± 3.65	193.23 ± 5.74
Means ± SD	206.14 ±6.36	203.39 ± 6.69	200.43 ± 6.43	196.81 ± 7.13	

Where, G_0 = Control Group (no prebiotic given), G_1 = 963mg/kg Inulin+10ppm NaFeEDTA, G_2 = 963mg/kg Inulin+20ppm NaFeEDTA, G_3 = 963 mg/kg GOS+15ppm $FeSO_4$, G_4 = 963mg/kg GOS+30ppm $FeSO_4$

Table 4: Effect of Fortified Diets on Immunoglobulin E (IU/mL) among Anemic Women

Treatments/ Groups	Days				Means ± SD
	0	30	60	90	
G_0	195.02 ± 1.06	192.59 ± 1.34	190.81 ± 1.32	187.32 ± 1.39	191.43 ± 3.24
G_1	184.86 ± 1.14	181.87 ± 2.19	179.00 ± 2.63	176.28 ± 3.49	180.50 ± 3.69
G_2	186.02 ± 2.81	182.36 ± 3.44	178.23 ± 3.79	174.09 ±3.50	180.17 ± 5.16
G_3	174.62 ± 1.59	171.73 ± 2.63	168.62 ± 3.31	164.60 ± 3.98	169.89 ± 4.30
G_4	193.76 ± 1.49	189.69 ± 2.47	185.56 ± 2.83	177.73 ± 3.90	186.68 ± 6.84
Means ± SD	186.85 ± 8.20	183.64 ± 8.11	180.44 ± 8.37	176.00 ± 8.14	

Where, G_0 = Control Group (no prebiotic given), G_1 = 963mg/kg Inulin+10ppm NaFeEDTA, G_2 = 963mg/kg Inulin+20ppm NaFeEDTA, G_3 = 963 mg/kg GOS+15ppm $FeSO_4$, G_4 = 963mg/kg GOS+30ppm $FeSO_4$

Production of GOS

First of all, lactose (600 mM) was synthesized in 50 mM NaPP (Tetra sodium pyrophosphate) buffer whose pH was adjusted at 6.5 and temperature at 370C. Next, transgalactosylation of lactose was done with the help of enzyme β-galactosidase at 300 revolutions per minute. The mixture was then heated at 90^0C for 5 minutes so as to cease this reaction. Samples were stored at -20^0C for subsequent procedure. Megazyme assay kits from Ireland were utilized to analyze glucose, galactose and lactose as per the standard procedure available in the manual. The calculation of GOS was performed making use of the formula;

GOS = Initial concentration of lactose - (Glucose + Galactose + Untransgalactosylated Lactose)

Thin Layer Chromatography (TLC) was then performed with the mixture of GOS to ascertain that the produced GOS was of high purity (Maawia *et al.,* 2016).

Study site

The current study was conducted in Islamabad, Pakistan.

Table 5: Effect of fortified diets on immunoglobulin G (mg/dL) among anemic women

Treatments/ Groups	Days				Means ± SD
	0	30	60	90	
G_0	1684.10 ± 2.39	1681.23 ± 2.12	1678.54 ± 2.46	1674.86 ± 1.86	1679.68 ± 3.94
G_1	1718.47 ± 3.49	1708.98 ± 5.18	1704.64 ± 5.80	1700.06 ± 6.47	1708.03 ± 7.85
G_2	1734.16 ± 2.61	1727.20 ± 3.66	1719.79 ± 4.51	1711.76 ± 4.32	1723.22 ± 9.64
G_3	1691.93 ± 2.76	1687.67 ±3.06	1682.53 ± 4.26	1678.98 ± 5.66	1685.27 ± 5.69
G_4	1707.56 ± 2.14	1684.95 ± 2.32	1655.01 ± 2.39	1622.73 ± 2.45	1667.67 ± 34.25
Means ± SD	1707.24±20.13	1698.00±19.57	1688.10±24.98	1677.67±34.25	

Where, G_0 = Control Group (no prebiotic given), G_1 = 963mg/kg Inulin+10ppm NaFeEDTA, G_2 = 963mg/kg Inulin+20ppm NaFeEDTA, G_3 = 963 mg/kg GOS+15ppm $FeSO_4$, G_4 = 963mg/kg GOS+30ppm $FeSO_4$

Table 6: Effect of Fortified Diets on Immunoglobulin M (mg/dL) among anemic women

Treatments/ Groups	Days				Means ± SD
	0	30	60	90	
G_0	303.66 ± 2.51	300.55 ± 2.51	299.38 ± 2.51	296.90 ± 2.51	300.12 ± 2.81
G_1	285.20 ± 2.57	281.25 ± 2.36	278.21 ± 2.59	271.64 ± 2.93	279.07 ± 5.72
G_2	273.74 ± 2.20	271.15 ± 2.31	267.81 ± 2.83	264.02 ± 3.15	269.18 ± 4.21
G_3	267.76 ± 3.05	264.41 ± 3.05	259.83 ± 3.23	256.95 ± 3.16	262.23 ± 4.79
G_4	256.37 ± 1.69	234.41 ± 1.87	213.71 ± 2.28	203.21 ± 3.43	226.92 ± 23.52
Means ± SD	277.34 ± 18.02	270.35 ± 24.28	263.78 ± 31.68	258.54 ± 34.41	

Where, G_0 = Control Group (no prebiotic given), G_1 = 963mg/kg Inulin+10ppm NaFeEDTA, G_2 = 963mg/kg Inulin+20ppm NaFeEDTA, G_3 = 963 mg/kg GOS+15ppm $FeSO_4$, G_4 = 963mg/kg GOS+30ppm $FeSO_4$

Study duration

It was a three months study that lasted from March 2019 to June 2019.

Study subjects

The study subjects for the current study involved University going iron deficient women (having age between 18 and 25 years).

Inclusion and exclusion criteria

Inclusion Criteria

All willing iron deficient women with no other medical conditions were made a part of study.

Exclusion criteria

Exclusion of subjects from the study was made on the basis of three criteria;

(i) married women (ii) women with other chronic diseases such as hypertension or diabetes (iii) women already taking prebiotic and/or iron supplements

Sampling

Sampling Technique

Technique of convenience sampling was used to recruit study subjects for instant research.

Sample Size and Grouping

G*Power software (University of Düsseldorf, Germany) was used to determine the sample size. The sample size was calculated with hypothesized effect size of 0.61, α error probability of 0.05 and power probability of 0.95. Total sample size calculated for the study came out to be as 75. Therefore, a total of seventy five women were chosen for the study and were randomly divided into five groups. One of the groups was control group while other four were treatment groups; each group had 15 study subjects.

Experimental protocol

For the current study, four different types of fortified wheat flour were prepared by adding iron fortificants and prebiotics to be given to treatment groups. For the control group, the flour was only fortified with iron without the addition of prebiotics. NaFeEDTA and FeSO4 were used as iron fortificants while Inulin and Galacto oligosaccharides were added as prebiotics. For NaFeEDTA, doses of 10 and 20 ppm were used while for $FeSO_4$, doses of 15 and 30 ppm were employed. In order to determine the dosage of prebiotics, Human Equivalent Dose (HED) equation was used (Reagan-Shaw *et al.,* 2008);

$$\text{HED (mg/kg)} = \text{Animal Dose in mg/kg} \times \frac{(\text{Animal Weight in kgs})^{0.33}}{(\text{Human Weight in kgs})}$$

Five groups therefore made, based on the provision of prebiotics and iron fortificants were as follows;

Bio-efficacy trials

Fortified flours were fed to experimental subjects daily for a period of 12 weeks. Blood samples were collected from overnight fasted women on monthly basis up to a period of three months.

Analytical Procedures
Immunoglobulins, including Immunoglobulin A, Immunoglobulin E, Immunoglobulin G, and Immunoglobulin M were determined using the technique of ELISA (Enzyme linked Immunosorbent Assay), as per their respective protocols (Lems-Van Kan *et al.,* 1983).

STATISTICAL ANALYSIS

Statistical analysis of obtained data was done using SPSS version 23.0. Factorial design was used in order to determine the level of significance (Steel and Torrie, 1997). Data was considered significant at P-value < 0.05. Results were presented as Means ± Standard Deviations. The assays were performed in triplicate and the results were expressed as Mean ± SD (standard deviation). Differences among means were evaluated by Tukey's test at P<0.05.

Ethical considerations
All procedures performed in studies involving human participants were in accordance with the ethical standards of the University of Veterinary & Animal Sciences, Lahore-Pakistan (approval taken from the Institutional Review Committee for Biomedical Research of the University with reference number 037/IRC/BMR dated February 04, 2019) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Additionally, informed consent was obtained from all individual participants included in the study.

RESULTS

According to the treatment plan followed during the trials (table 1), group G_0 was the control group which was given 20 ppm NaFeEDTA without any prebiotics. Group G_1 was given 963 mg/kg Inulin and 10 ppm NaFeEDTA while group G_2 was given 963 mg/kg Inulin and 20 ppm NaFeEDTA. Group G_3 was provided with 963 mg/kg GOS along with 15 ppm FeSO4 while group G_4 was fed with 963 mg/kg GOS along with 30 ppm $FeSO_4$.

Mean square values for Immunoglobulin A, Immunoglobulin E, Immunoglobulin G, and Immunoglobulin M showed that significant variations existed for the effect of groups, study intervals as well as their interaction (table 2).

Immunoglobulin A
Mean values for Immunoglobulin A levels along with their respective standard deviations have been shown in table 3. The table depicts that among the treatment groups, maximum value of 210.47±3.97mg/dL was observed in group G_1, followed by value of 205.45±4.46mg/dL in G_2 and 200.01±3.79mg/dL in G_3. Minimum value for this trait was observed in group G_4 which was 193.23±5.74mg/dL. Control group G_0 was observed to have a value of 199.30±2.26mg/dL.

Immunoglobulin E
As per the mean values of Immunoglobulin E, it can be observed that highest value was witnessed in group G_4 (186.68±6.84IU/mL), followed by group G_1 (180.50±3.69IU/mL) and group G_2 (180.17±5.16IU/mL). Lowest value for the trait was observed in group G_3 (169.89±4.30IU/mL) while for the control group G_0, the recorded value was 191.43±3.24IU/mL (table 4).

Immunoglobulin G
Mean values for Immunoglobulin G shown in table 5 clearly indicate that lowest value was attained by group G_4 (1667.67±34.25mg/dL). Highest value for the trait was attained by group G_2 (1723.22±9.64mg/dL). Control group G_0 had a mean value of 1679.68±3.94mg/dL while group G_1 was recorded having a value of 1708.03±7.85mg/dL. Group G_3 had a mean value of 1685.27±5.69mg/dL for Immunoglobulin G.

Immunoglobulin M
Amongst the groups, highest value was observed in control group G_0 (300.12±2.81mg/dL), followed by groups G_1 (279.07±5.72mg/dL), G_2 (269.18± 4.21mg/dL), G_3 (262.23±4.79mg/dL) and G_4 (226.92± 23.52mg/dL).

DISCUSSION

Among placental mammals, Immunoglobulins are categorized into 5 major classes, based on the isotype, each of which has a specific function as well as response to antigens mainly due to difference in structure. The five major classes of immunoglobulins include IgA, IgD, IgE, IgG and IgM (Parker *et al.,* 2016).

IgA comprises the primary antibody class which is actually the first line of defence in the body (Kilian and Russell, 2015). IgE is only present in small amounts in humans and has the major function of providing immunity against parasites, mainly Helminths (Oettgen, 2016). IgG is the most abundant antibody in blood plasma and it helps detoxify harmful substances entering in the body (Irani *et al.,* 2015). IgM accounts for approximately 10% of total human immunoglobulins and is the first antibody released by the body in order to fight off an infection (Wang *et al.,* 2016).

Several micronutrients such as iron, zinc, copper, selenium, Vitamin A, Vitamin C, Vitamin E and others have known to impact innate immunity of an individual. These micronutrients are therefore required in adequate quantities so as to maintain the appropriate immune function of an individual and any deficiencies would have a negative impact on the immunity of an individual. Supplementation or fortification of the respective

deficient micronutrients however can help regain the normal immune function (Núñez *et al.,* 2018).

There is definite evidence that the immune profile of patients suffering from Iron Deficiency Anemia is altered but the exact nature of alteration is still unknown. It is believed that immunoglobulins are released in increased amounts in anemic individuals but the precise role of iron with regards to immuno-regulatory response continues to be unclear (Mullick *et al*., 2006).

In our study, we hypothesized that with the provision of prebiotic and iron fortified wheat flour to iron deficient women, production of Immunoglobulins would be decreased, indicating that the symptoms of Iron Deficiency Anemia are being gradually alleviated. Our study results showed that as anemic women consumed fortified flour, all the four types of Immunoglobulins under consideration were decreased which indicated that fortified flour was helpful in alleviating the symptoms of Iron Deficiency Anemia.

Mullick *et al.* conducted a study to report the changes in T cell subsets among iron deficient under 5 children. The results of the study suggested that lower levels of T Lymphocytes and CD4+ cells were present in iron deficient children (P-value <0.05). Moreover, they also found that CD4 to CD8 ratio was also lower in patient group (P-value <0.05). The researchers concluded that supplementation of iron significantly helped improve the CD4 counts among anemic children (Mullick *et al.,* 2006). These results are in harmony with our study results as we have also reported that provision of prebiotic and iron fortified flour significantly improved the immune function of the patients.

Effect on Immunoglobulin A & E Levels

In our study, with the progression of study intervals, it was observed that a considerable decrease in mean Immunoglobulin A levels was recorded. Maximum decline was observed in group G_4, followed by groups G_2, G_1 and G_3. For group G_4, the mean value changed from 199.97±1.34mg/dL at 0 day to 186.22±3.65mg/dL at 90th day while for group G_2, the mean value declined from 210.56±1.26mg/dL at the start to 200.37±4.67mg/dL at the end of trials. In group G_1, the value of the trait ranged from 214.75±1.39mg/dL at baseline to 205.58 ± 2.75mg/dL at the termination. In group G_3, the value of the trait was 204.54±1.31mg/dL at 0 day and it declined to 195.68±3.22mg/dL at 90th day.

During the course of efficacy trials, a significant decline in mean Immunoglobulin A levels was noticed, ranging from 206.14±6.36mg/dL at baseline to 203.39± 6.69mg/dL, 200.43±6.43mg/dL and 196.81±7.13mg/dL at 30th, 60th and 90th day, respectively.

For Immunoglobulin E, in terms of progression of study trials, it was observed that a considerable decrease existed across all the study groups. Maximum decrease occurred in group G_4 whose value ranged from 193.76±1.49IU/mL at the start to 177.73±3.90IU/mL at the end of trials. Subsequent decline was observed in groups G_2, G_3 and G_1. In group G_2, the mean value of Immunoglobulin E ranged from 186.02±2.81IU/mL at baseline to 174.09±3.50IU/mL at 90th day. For groups G_3 and G_1, the mean values ranged from 174.62±1.59IU/mL and 184.86±1.14IU/mL at 0 day to 164.60±3.98IU/mL and 176.28±3.49IU/mL at 90th day, respectively.

During the modeling trials, a considerable decrease in Immunoglobulin E levels was witnessed which varied from 186.85±8.20IU/mL at 0 day to 183.64±8.11IU/mL at 30th day. When the trials were further progressed, the value subsequently declined to 180.44±8.37IU/mL at 60th day and to 176.00±8.14IU/mL at 90th day.

Effect on Immunoglobulin G & M Levels

For Immunoglobulin G, amongst the groups, maximum decrease for the trait was observed in group G_4, followed by groups G_2, G_1 and G_3, respectively. In group G_4, the level varied from 1707.56±2.14mg/dL at baseline to 1622.73±2.45mg/dL at termination. Group G_2 had a mean value of 1734.16±2.61mg/dL at 0 day which was declined to 1711.76±4.32 mg/dL at 90th day. Furthermore, groups G_1 and G_3 had values of 1718.47±3.49mg/dL and 1691.93±2.76 mg/dL at baseline which decreased to 1700.06±6.47 mg/dL and 1678.98±5.66 mg/dL, respectively.

During the modeling trials, when women were fed with prebiotic and iron fortified flour, a significant decline in mean values of Immunoglobulin G was observed. The value for the trait was recorded to be 1707.24± 20.13mg/dL at 0 day which was decreased to 1698.00± 19.57mg/dL at 30th day. Further, the trait was declined to 1688.10±24.98mg/dL at 60th day and to 1677.67± 34.25mg/dL at 90th day.

With regards to Immunoglobulin M, maximum decline was observed in group G_4, which varied from 256.37±1.69mg/dL at 0 day to 203.21±3.43mg/dL at 90th day. Subsequent decline was observed in group G_1, ranging from 285.20±2.57mg/dL at baseline to 271.64± 2.93mg/dL at the termination of trials. For group G_3, the mean values varied from 267.76±3.05mg/dL at the start to 256.95±3.16mg/dL at the end of trials while for group G_2, recorded decline was from 273.74± 2.20mg/dL at initiation to 264.02±3.15mg/dL at termination.

Across the modeling trials, the mean value of Immunoglobulin M varied from 277.34±18.02mg/dL at 0 day to 270.35±24.28mg/dL, 263.78±31.68mg/dL and 258.54±34.41mg/dL at 30th, 60th and 90th day,

respectively. A study was conducted by Tang *et al.* in which the researchers evaluated the immune system changes among pregnant women with Iron Deficiency Anemia. The study results indicated that serum IgG levels, the ratio of CD4+ to CD8+ cells and the levels of CD3+ and CD4+ cells were significantly lower among anemic pregnant women, compared to non anemic pregnant women (P-value <0.05). The study concluded that development of Iron Deficiency Anemia did have effects on immune function of anemic women during pregnancy (Tang *et al.,* 2006). Our study results have also shown similar findings as we have also confirmed statistically significant changes in the production of Immunoglobulins among anemic women of childbearing age.

In another research, the peers studied the effects of iron deficiency anemia on immunity among children. They concluded that activity of cytokines, cell mediated, non specific and humoral immunity, all are impacted by the development of Iron Deficiency Anemia (Ekiz *et al.,* 2005). In a similar study, the researchers studied humoral immunity among pregnant anemic women. They found out that B-system immunity was suppressed as anemia progressed. Moreover, they also reported that among anemic pregnant women, IgG and IgM levels significantly increased (Leush and Futornyĭ, 1997). These results are also in accordance with our study results since we have also reported that levels of IgG and IgM were significantly decreased with the rectification of Iron Deficiency Anemia.

In another attempt, the researchers studied the relationship between iron deficiency, morbidity and cell mediated immunity among children in Togo. The study results concluded that an inverse relationship existed between number of helper inducer T and mature T lymphocytes and that of iron status of children (Berger *et al.,* 1992).

Sadeghian *et al.* conducted a similar study which aimed at determining if serum immunoglobulins would change in anemic adult women or not. It was a case control study comprising of 45 anemic patients and 45 controls. Serum IgA, IgG and IgM were determined and compared in both the groups. The results of the study found that serum immunoglobulins were somewhat decreased in patients, compared to the control group however this difference was not statistically significant (Sadeghian *et al.,* 2010). The results of another research study concluded that iron deficiency anemia impairs immune function of patients and may put them at an increased risk of acquiring infections and developing different diseases (Ahluwalia *et al.,* 2004).

CONCLUSION

The current study concluded that prebiotics coupled with iron fortificants showed significant immunomodulatory effects with regards to Immunoglobulin A, E, G and M. This reveals that prebiotics could well play a very important role in improving the immune status of patients suffering from iron deficiency anemia. It is also now believed from recently conducted researchers that prebiotics help enhance the absorption of iron. Therefore, a recommendation for anemic patients would be to consume prebiotics along with iron so as to have increased iron absorption as well as improved immune system.

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