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A PROSPECTIVE COMPARITIVE STUDY ON PROBIOTIC: TO ENHANCE THE BIOAVAILABILITY OF IRON IN YOUNG FEMALE ADULTS

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ABSTRACT

Anemia is strictly defined as a decrease in red blood cell (RBC) mass. According to the World Health Organization (WHO), anemia is defined as hemoglobin (Hb) levels <12.0 g/dL in women and <13.0 g/dL in men. The function of the RBC is to deliver oxygen from the lungs to the tissues and carbon dioxide from the tissues to the lungs. Methods for measuring RBC mass are time consuming and expensive and usually require transfusion of radiolabeled erythrocytes. Thus, in practice, anemia is usually discovered and quantified by measurement of the RBC count, Hb concentration, and hematocrit. The aim and objective of study is to identify the subjects who are anemic, to assess the impact of patient counselling for anemic patients by providing them iron rich diet, folic acid supplements and probiotic capsules .Estimation of blood parameters [Hb%, Iron content].To observe the impact of probiotics in enhancing the iron absorption. It is a prospective comparative study on 80subjects in female of age groups 18-25yrs during three month period. After 12 weeks of treatment, there was significant difference in the increase in serum iron taking the probiotic LP299V compared with controls {Control: 80.6mg/dl to 79.99mg/dl,Diet: 50.05mg/dl to 51.1mg/dl, Folic acid+Diet: 45.15mg/dl to 48.35mg/dl, Probiotic+Folic acid+Diet: 64.6mg/dl to 74.1mg/dl}. Additionally, an increase in iron level was significantly associated with probiotics and folic acid use. This study finds only about 10-15% of the iron ingested through the normal diet is absorbed by the body. This may have big impact on ironlevels leading to anemia common in women of child bearing age. Iron absorption concept based on LP229V in combination with iron and folic acid is clinically proven to increase iron uptake.

INTRODUCTION

Anemia is strictly defined as a decrease in red blood cell (RBC) mass. According to the World Health Organization (WHO), anemia is defined as hemoglobin (Hb) levels <12.0 g/dL in women and <13.0 g/dL in men. Basically, only three causes of anemia exist such as blood loss, increased destruction of RBCs (hemolysis), and decreased production of RBCs.. India has among the highest number of cases of anemia in the world, according to the NFHS-III undertaken in 2005-2006. The reasons range from high cost of healthcare facilities, poor food quality and the low status of women. [1-20]

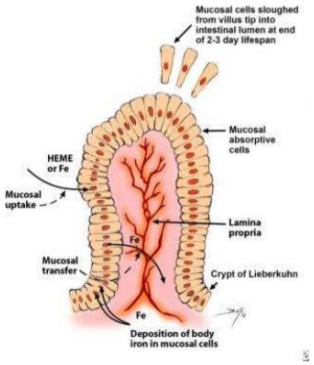
Signs and symptoms

Patients with iron deficiency anemia may report the following:

Fatigue and diminished capability to perform hard labor

- Poor scholastic performance
- Cold intolerance
- Reduced resistance to infection
- Altered behavior (eg: attention deficit disorder)
- Dysphagia with solid foods (from esophageal webbing)
- Worsened symptoms of comorbid cardiac or pulmonary disease

Pathophysiology:



PROBIOTICS:

The World Health Organization (WHO) and Food and Agricultural Organization of the United Nations (FAO) have together stated a definition of probiotics. Probiotics are "Live microorganisms which when administrated in adequate amounts confer a health benefit on the host". WHO and FAO have some guidelines for probiotics. A food product has to contain a sufficient amount of microorganisms that also are alive when the product is supposed to be consumed, to be called probiotic. Also the microorganism should be able to survive the tough environment in the stomach. Beneficial probiotic properties are strain specific. Human studies should always be the basis of the probiotic health benefit. [20-25]

Name of the GRAS Organism: The subject of this Generally Recognized as Safe (GRAS) determination is a strain of the probiotic bacterium Lactobacillus plantarum designated 299v. The strain is also known commercially as Plantarurn 299v and Lp299v. [26]

Source of the GRAS Organism:L. plantarum299v was isolated from healthy intestinal mucosa .It was isolated from a biopsy taken from a patient with polyps, but the biopsy was taken from healthy mucosa.

Description of the GRAS Organism: L.Plantarum is a Gram-positive, catalase-negative, bacterium that is a member of the broad classification of lactic acid bacteria (LAB). LAB comprises a group of microbes related by common metabolic functionality-the production of lactic acid as the major metabolic end product of carbohydrate metabolism-and common physiological traits. LAB are Gram-positive, non-spore-forming, and catalase-negative and are devoid of cytochromes. They are preferential non aerobes but are aero tolerant, acid-tolerant, and strictly fermentative.

Mechanism of Lactobacillus plantarum in non-heme iron absorption: Lactobacillus plantarum299v is a probiotic strain hypothesized to have a positive outcome for iron absorption. The plausible mechanism has previously been described as an effect of the low pH and organic acids. The low pH can prevent the formation of complexes with low solubility and also activate phytates. The organic acids can chelate with iron and keep it in solution so it is more absorbable. Organic acids may also delay the gastric emptying that increases the iron exposure to the proximal intestinal epithelium which might lead to increased iron absorption. [27]

The role of vitamin c in iron absorption: Iron requirements remain the same despite the current lower energy requirement. This means that more iron must be absorbed per unit energy. A higher bioavailability of the dietary iron can be achieved by increasing the content of food components enhancing iron absorption (ascorbic acid, meat/fish) or by decreasing the content of inhibitors (e.g., phytates, tannins). The latter is not feasible considering the recent and reasonable trend toward increasing the intake of dietary fiber. The key role of ascorbic acid for the absorption of dietary nonheme iron is generally accepted. The reasons for its action are twofold: (1) the prevention of the formation of insoluble and unabsorbable iron compounds and (2) the reduction of ferric to ferrous iron, which seems to be a requirement for the uptake of iron into the mucosal cells.

FOLATES: Folates are hydrophilic anionic molecules that do not cross biological membranes by diffusion, but specialized membrane transport systems allow folate accumulation into mammalian cells and tissues. Absorption exploits several genetically and functionally distinct transporters, such as the folate receptors, the family of organic anion transporters, a proton-coupled folate transporter, and the reduced folate carrier, which is ubiquitously expressed.[28] Each mechanism plays a unique role in mediating the transport across epithelia and into systemic tissues, and contributes to folate homeostasis in humans. [29] Even though absorption occurs primarily in the duodenum and upper jejunum, the colon represents a major depot of folate and the vitamin produced by the colonic bacteria exceeds dietary intake and affects the folate status of the host. It is produced in large quantities by the colonic microbiota, mainly as monoglutamylated folate, the form that is absorbed at the highest rate, [30] intestinal bacteria being one source of this vitamin. Many studies assessed the contribution of intestinal microbiota to the folate intake of animal hosts [30,31,32,33], and it has been demonstrated that the folate synthesized by intestinal bacteria can be absorbed and used by the host.[34,35] Recently, direct evidence of absorption of folate across the colon has been irrefutably provided. [36] The apparent rate of absorption in the colon is considerably lower than that in the small intestine. However, in the distal portion of the gastrointestinal tract the transit time is longer than in the small intestine, and the supply of folates by the colonic microbiota is constant and continuous, whereas their availability in the upper tract is discontinuous and mostly affected by food intake.

METHODOLOGY:

Study design: It is a prospective-comparative study

Sample size: 80 subjects

Inclusion criteria: Those who are willing to participate in the study

Females in the age group of 18-25yrs

Exclusion criteria: Male subjects

Those who are not willing to participate in the study

Study site: Nirmala College of Pharmacy

Study duration: Three month period.

Informed consent forms were distributed to the students between the age group 18-25yrs. detailed information of our study (project name, aim & objective of our study, selection criteria, supplementation of folic acid and probiotics) was explained and those who were willing to participate in the study were selected and were asked to sign the informed consent form.

- Iron rich diet chart was prepared which was approved by the Dietician from Manipal hospital and diet chart was provided to the subjects in diet group after explaining the beneficial outcomes of following the diet and regular follow up was done.
- Iron and folic acid supplements were provided to the 3rdgroup, 1tab/week and were advised to take after breakfast.
- To the 4th group Probiotic capsules (Lpv299v) were provided and were advised to take every alternative day after lunch along with iron and folic acid 1tab/week after breakfast.
- Diet chart was provided to both 3rd and 4th groups
- Monitoring was done on the same day when the supplementations were provided and complaints reported by subjects were noted and the reasons were identified.
- Further another sample was collected after one month and Hb% along with Fe content was calculated from the obtained blood samples. Based on their results the level of improvement in Hb% between different treatment groups was compared.

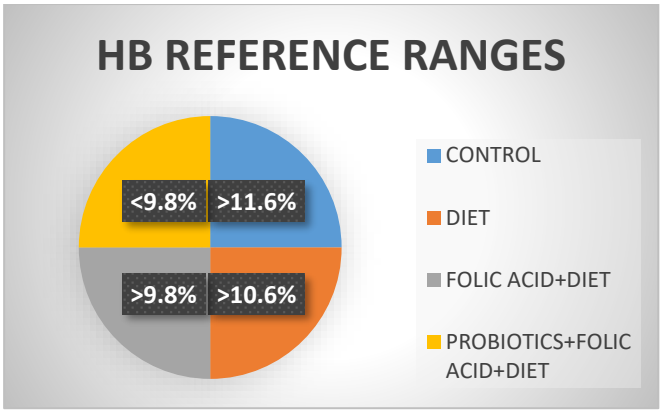


Figure 1: Pie chart representing different treatment groups and their selection criteria

SUNRISE HOSPITALS

INSTITUTIONAL ETHICAL COMMITTEE

The committee has looked into the proposed *in vivo* studies on the research topic of **"PROBIOTIC: TO ENHANCE THE BIOAVAILABILITY OF IRON IN YOUNG FEMALE ADULTS"**, to be done by **Ms.NANDYALA.BHARGAVI, Ms.KOTHAMASU SWETHA and Mr .GUNDALA VENKATA NAVEEN KUMAR**, students of 5th Year Pharm.D , Department of Pharmacy Practice, Nirmala College of Pharmacy, Atmakur, Guntur, Andhra Pradesh, under the guidance of **Prof. Shanta Kumari Adiki**, Nirmala College of Pharmacy, Atmakur, Guntur, Andhra Pradesh and has been allotted the following IEC Re-registration Number ECR/756/Inst/AP/2015/RR-18 issued under Rule 122DD of Drugs and Cosmetics Rules 1945, Dated: 03rd September, 2018 for future reference.

The committee is in complete perspective and is of the conscientious opinion that the study can be conducted with a mandatory WRITTEN INFORMED CONSENT from the subjects who have volunteered for the study to avoid legal, moral and ethical encumbrances. It is pertinent to conduct the study under the strict supervision of the guide. Any complications arising shall be referred to the managed by staff physician who is also a member of the expert panel. The inclusion and exclusion criteria, method of selection of the patients, ramification, and expected complications review of literature have been critically analysed and consent given for proceeding with the study. The scientific study is of the current norms for research work in clinical studies.

Chairman

SUNRISE HOSPITAL VJA-2.

Date: 03rd September, 2018

S.No	NAME	Sample-1	Sample-2	
		Hb%		Iron content(mg/dl)
	CONTROL			
1	A	12	10	47
2	B	12	11.8	52
3	C	11.8	11.8	47
4	D	12	12	51
5	E	11.8	11.4	121
6	F	11.6	9.2	52
7	G	12	11.6	155
8	H	11.8	11.1	49
9	I	11.6	11.6	50
10	J	12	12	55
11	K	12	11.8	79
12	L	11.6	11.6	67
13	M	12	12	55
14	N	12	11.6	76
15	O	12	12	133
16	P	11.8	11.8	78
17	Q	11.6	11.4	86
18	R	12	12	145
19	S	11.6	11.4	120
20	T	11.6	11.6	95
	DIET			
21	A1	11	11	30
22	B1	10.8	10.6	21
23	C1	10.2	10.6	38
24	D1	10.8	11	35
25	E1	11.2	11.2	68
26	F1	11.2	11	53
27	G1	11.4	10.4	21
28	H1	11.2	9.8	52
29	I1	11	10.2	92
30	J1	10.8	9	40
31	K1	11.4	9.5	45
32	L1	11	9.2	52
33	M1	10.8	10.8	45
34	N1	10.8	10.8	42
35	O1	11	11.6	50
36	P1	11.4	11.2	52
37	Q1	10.8	11	63
38	R1	10.2	10.2	49
39	S1	11.2	11	82
40	T1	11	10.8	71

	FOLIC ACID+DIET			
41	A2	10.4	10.2	26
42	B2	10.6	10.6	21
43	C2	10	10.2	32
44	D2	10	10.6	38
45	E2	10.4	10.2	28
46	F2	10.6	10.6	64
47	G2	10.2	10	30
48	H2	10.4	10	28
49	I2	10.6	10.6	22
50	J2	10.4	10.2	28
51	K2	10.6	10.6	25
52	L2	10	10.2	35
53	M2	10	10.4	40
54	N2	10.6	10.6	56
55	O2	10.2	10.2	62
56	P2	10.6	10.8	70
57	Q2	10.4	10.2	79
58	R2	10	10.6	82
59	S2	10.2	10.2	59
60	T2	10.2	10	78
	PROBIOTICS+FOLIC ACID+DIET			
61	A3	6	6.6	30
62	B3	3.6	6.2	44
63	C3	8.4	8.4	39
64	D3	4.6	8.2	42
65	E3	5.2	5	22
66	F3	8.8	8.8	51
67	G3	8	8.6	78
68	H3	9.2	9.2	82
69	I3	6.6	8	90
70	J3	9.8	10.4	72
71	K3	8.4	8.4	85
72	L3	4.6	6.2	95
73	M3	6	8	89
74	N3	9.4	10	70
75	O3	7.6	8	68
76	P3	8.2	8.2	65
77	Q3	8.2	8.8	77
78	R3	6.8	7	74
79	S3	9	11.2	64
80	T3	9	9	55

TABLE 1: Hb% and Iron values before and after the treatment

RESULTS AND DISCUSSION

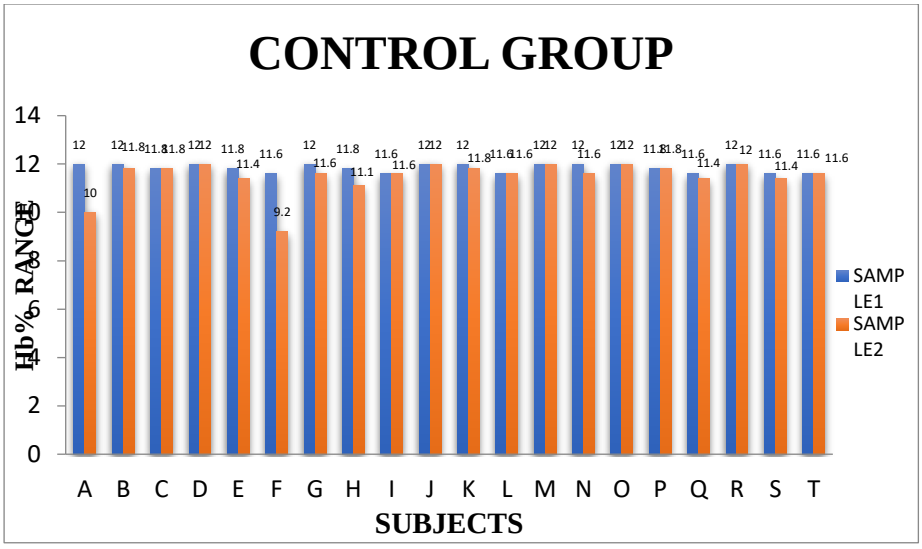


FIGURE 2: The graph shows the difference between Hb values taken from the control group. It was observed that 50 %(n=10) of the subjects showed no difference while in the remaining 50% (n=10) of the subjects the value decreased.

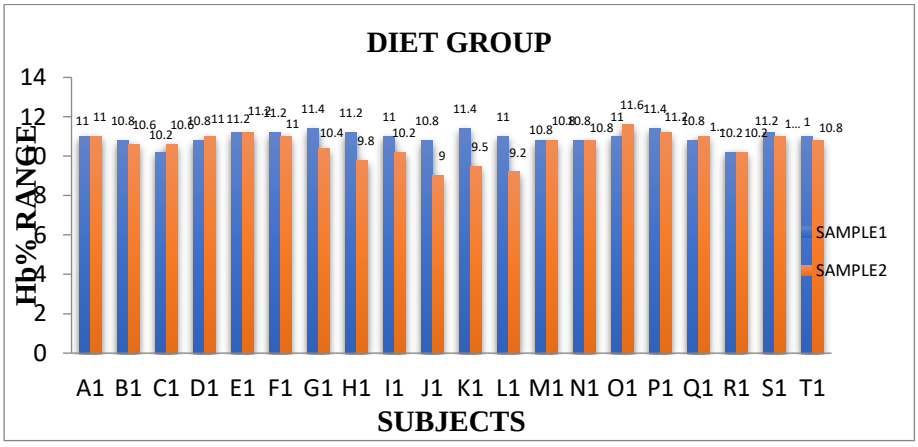


FIGURE 3: The above graph shows the difference between Hb% before and after following an iron rich diet. It was observed that 25 %(n=5) of the subjects showed an increase in their Hb content, 50 %(n=10) of the subjects the value decreased while the remaining 25 %(n=5) had no difference.

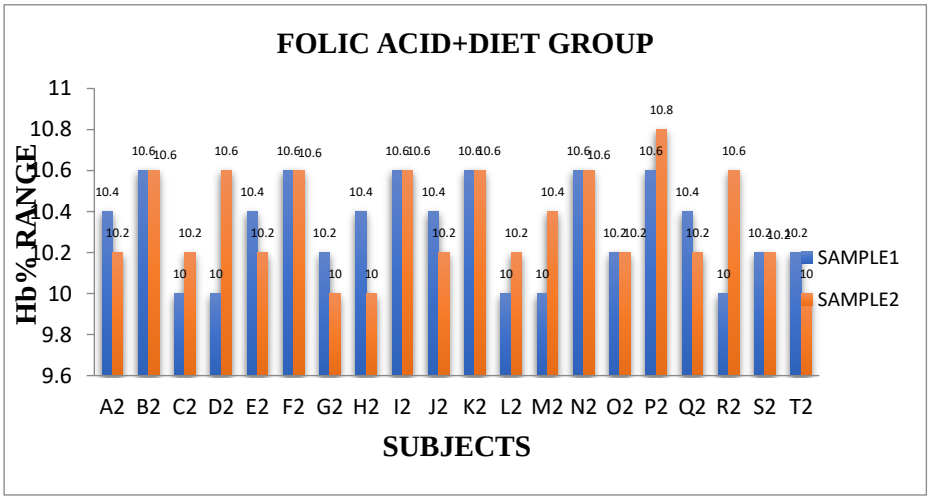


FIGURE 4: The above graph compares the Hb values before and after taking the supplements. It was observed that 30 %(n=6) of the subjects had an increased Hb value, 35 %(n=7) showed reduced Hb value while the remaining 35 %(n=7) showed no difference.

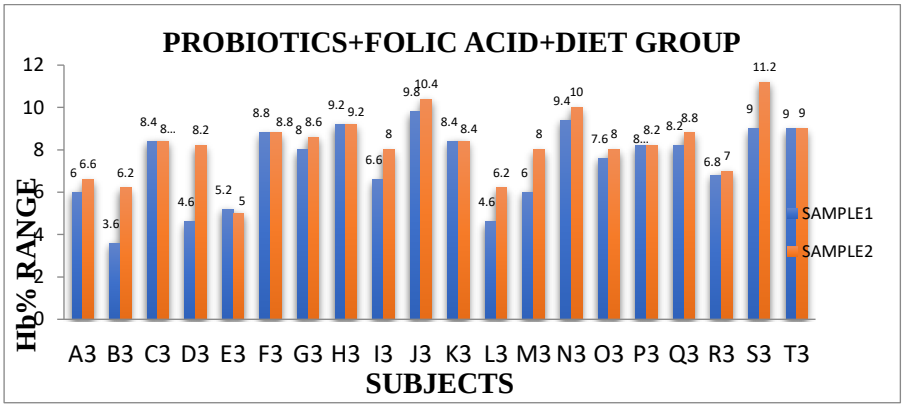


FIGURE 5: The above graph shows the difference between the Hb% before and after taking the supplements along with diet. It was observed that in 65 %(n=13) of the subjects the Hb values were increased, 30 %(n=6) showed no difference and in the remaining 5 %(n=1) Hb content was decreased.

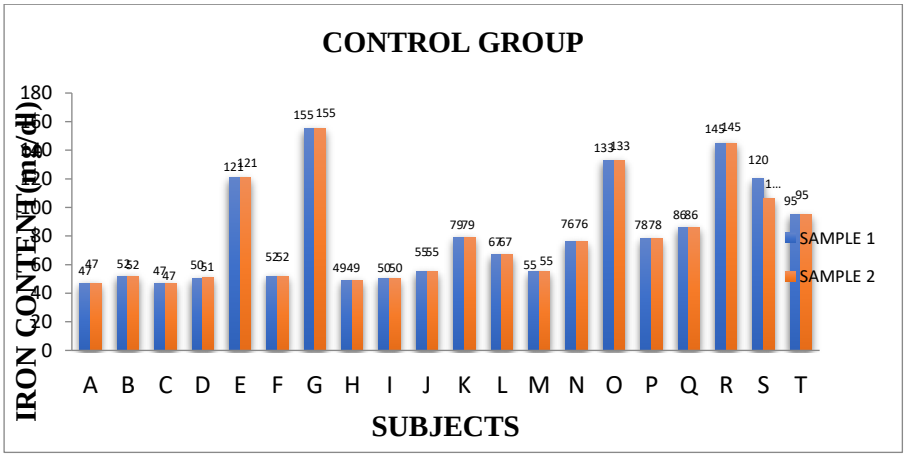


FIGURE 6: The above graph shows the difference in iron values between the two blood samples taken from the control group. It was observed that 90 %(n=18) of the subjects showed no significant difference and the remaining 10 %(n=2) showed reduced iron values.

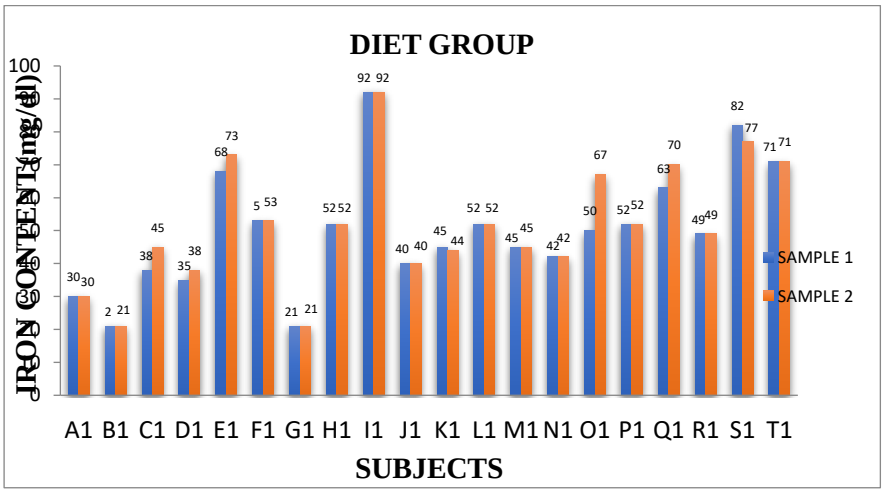


FIGURE 7: The above graph represents the difference in iron content before and after following the diet. It was observed that 25 %(n=5) of the subjects showed increased iron content, 10 %(n=2) showed reduced values while the remaining 65 %(n=13) showed no difference

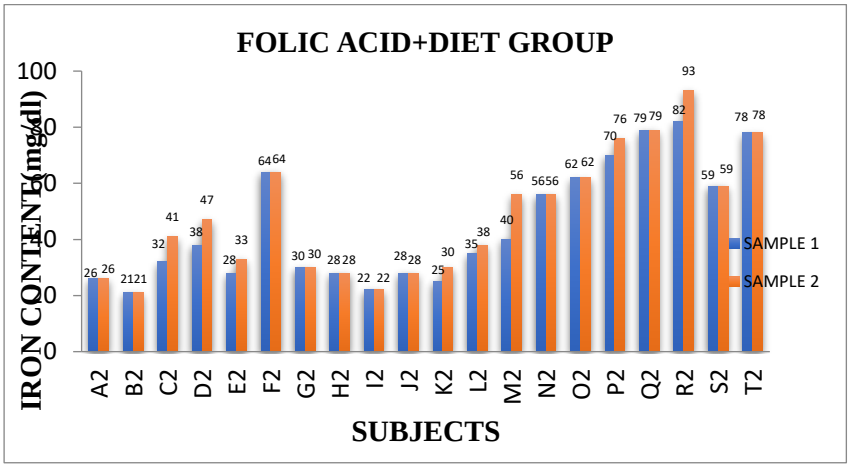


FIGURE 8: The above graph represents the iron values in the subjects who are taking the supplements. It was observed that 40 %(n=8) of the subjects had an improvement in the iron content while the remaining 60 %(n=12) showed no difference.

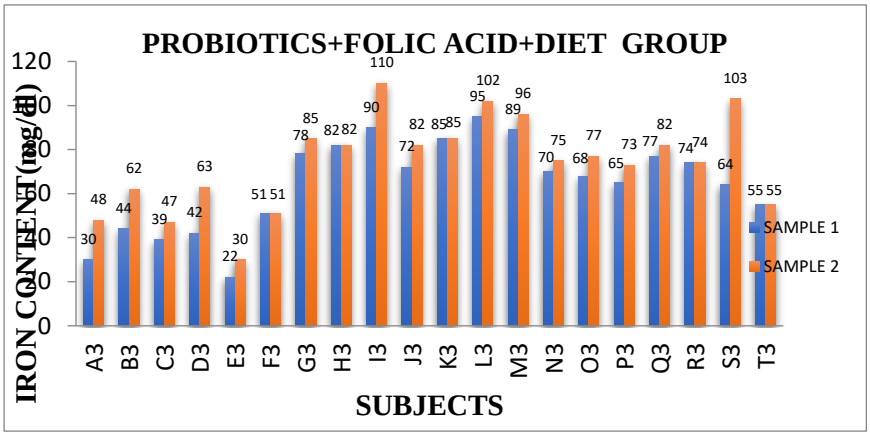


FIGURE 9: The above graph represents the difference in the iron values before and after taking the supplements. It was observed that in 75 %(n=15) of the subjects there was an improvement in the iron content while the remaining 25 %(n=5) showed no difference.

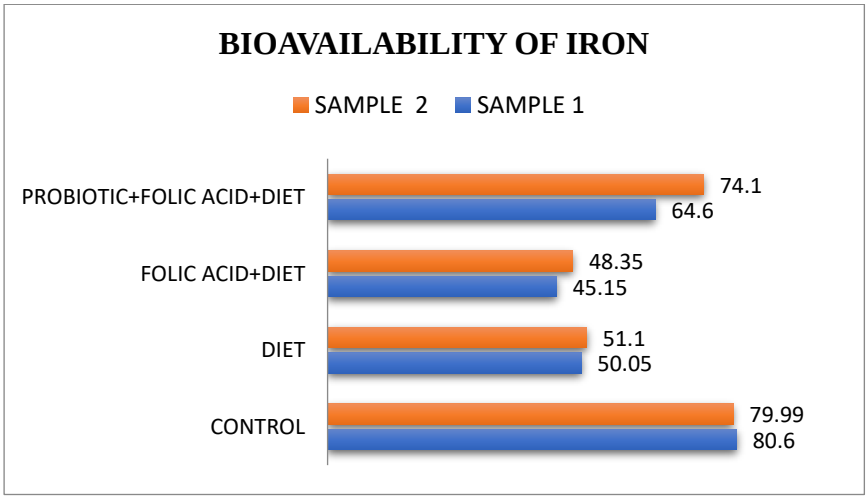


Figure 10: The above graph compares the average of iron content in all the four treatment groups. It was observed that that probiotic +folic acid+ diet group showed significant increase in the iron content when compared to the other treatment groups.

ANOVA for Quadratic model

Response 1: Hemoglobin

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	0.8207	9	0.0912	31.02	< 0.0001	significant
A-Diet	0.0840	1	0.0840	28.59	0.0011	
B-Folic acid	0.0512	1	0.0512	17.41	0.0042	
C-Probiotic	0.3528	1	0.3528	120.00	< 0.0001	
AB	0.0342	1	0.0342	11.64	0.0113	
AC	0.0420	1	0.0420	14.29	0.0069	
BC	0.2162	1	0.2162	73.55	< 0.0001	
A^2	0.0362	1	0.0362	12.32	0.0099	
B^2	0.0044	1	0.0044	1.49	0.2618	
C^2	0.0013	1	0.0013	0.4262	0.5347	
Residual	0.0206	7	0.0029			
Lack of Fit	0.0199	3	0.0066	39.02	0.0020	significant
Pure Error	0.0007	4	0.0002			
Cor Total	0.8413	16				

ANOVA for Linear model

Response 2: Iron content

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	51.28	3	17.09	12.98	0.0003	significant
A-Diet	2.88	1	2.88	2.19	0.1630	
B-Folic acid	9.68	1	9.68	7.35	0.0178	
C-Probiotic	38.72	1	38.72	29.41	0.0001	
Residual	17.12	13	1.32			
Lack of Fit	17.09	9	1.90	271.26	< 0.0001	significant
Pure Error	0.0280	4	0.0070			
Cor Total	68.40	16				

Solutions

47 Solutions found

Number	Diet	Folic acid	Probiotic	Hemoglobin	Iron content	Desirability	
1	**59.737**	**10.000**	**10.000**	**0.729**	**7.175**	**0.826**	**Selected**
2	58.841	10.000	10.000	0.727	7.171	0.826	
3	60.305	10.000	10.000	0.731	7.178	0.826	
4	57.873	10.000	10.000	0.723	7.166	0.826	
5	61.346	10.000	10.000	0.734	7.183	0.826	
6	61.909	10.000	10.000	0.736	7.185	0.826	
7	56.929	10.000	10.000	0.720	7.161	0.826	
8	64.324	10.000	10.000	0.744	7.197	0.826	
9	51.886	10.000	10.000	0.704	7.137	0.826	
10	68.111	10.000	10.000	0.755	7.215	0.826	
11	55.500	10.000	9.990	0.715	7.150	0.825	
12	49.872	10.000	10.000	0.698	7.128	0.825	
13	54.134	10.000	9.985	0.710	7.142	0.825	
14	47.737	10.000	9.999	0.691	7.117	0.825	
15	43.082	10.000	10.000	0.675	7.095	0.824	
16	63.493	9.962	10.000	0.739	7.185	0.824	
17	42.162	10.000	10.000	0.672	7.091	0.824	
18	40.052	10.000	10.000	0.665	7.080	0.823	
19	37.809	10.000	10.000	0.657	7.070	0.823	
20	81.991	10.000	10.000	0.797	7.282	0.823	
21	36.463	10.000	10.000	0.653	7.063	0.822	
22	35.152	10.000	10.000	0.648	7.057	0.822	
23	85.194	10.000	10.000	0.806	7.297	0.822	
24	56.243	9.911	10.000	0.713	7.139	0.822	
25	32.191	10.000	10.000	0.638	7.043	0.821	
26	70.811	9.910	9.991	0.757	7.204	0.821	
27	67.389	9.886	10.000	0.746	7.187	0.820	
28	28.648	10.000	10.000	0.626	7.026	0.819	
29	57.096	10.000	9.842	0.708	7.093	0.819	
30	96.678	10.000	10.000	0.838	7.352	0.817	
31	56.070	10.000	9.762	0.698	7.053	0.816	
32	67.710	9.782	10.000	0.740	7.165	0.815	
33	70.424	10.000	9.745	0.740	7.114	0.815	
34	51.798	10.000	9.727	0.681	7.017	0.814	
35	64.935	9.748	10.000	0.729	7.144	0.814	
36	52.797	9.753	10.000	0.691	7.087	0.814	
37	99.616	10.000	9.937	0.840	7.339	0.813	

38	102.064	10.000	10.000	0.852	7.378	0.811
39	90.884	10.000	9.717	0.797	7.200	0.809
40	7.098	10.000	10.000	0.547	6.922	0.807
41	72.696	9.601	10.000	0.742	7.149	0.806
42	56.926	10.000	9.498	0.678	6.941	0.805
43	0.000	9.979	10.000	0.519	6.884	0.800
44	47.406	10.000	9.228	0.626	6.776	0.792
45	0.000	9.684	10.000	0.502	6.819	0.785
46	4.937	9.238	10.000	0.496	6.744	0.766
47	12.497	9.076	10.000	0.514	6.745	0.764

IRON TESTING PROCEDURE IN BLOOD SAMPLES BY FERROZINE METHOD WITHOUT DEPROTEINIZATION

METHOD PRINCIPLE: Colorimetric method with ferrozine without deproteinization. Iron ions (Fe^{+3}), bounded to transferrin in blood is released in acid solution by detergents and reduced to Fe^{2+} by hydroxylamine hydrochloride. Fe^{2+} forms with 3-(2-pyridyl)-5, 6-bis (2-[4-phenyl sulfonic acid])-1, 2, 4-triazine sodium salt (ferrozine) coloured complex. Cu^{2+} ions are bound to (neokuproine-HCl). The colour intensity is directly related to the iron concentration.

REAGENTS: Working reagent preparation and stability Assay can be performed with use of separate 1-FERRUM and 2-FERRUM reagents or with use of working reagent. For working reagent preparation mix gently 4 parts of 1-FERRUM with 1 part of 2-FERRUM. Avoid foaming. Stability of working reagent: 4 weeks at 2-8°C, 2 weeks at 15-25°C . Concentrations in the test sodium acetate (pH 4.5) 100mmol/l ,hydroxylamine hydrochloride 220mmol/l, acetic acid 100mmol/l ,neokuproine hydrochloride > 2mmol/l, ascorbic acid 4mmol/l ,3-(2-pyridyl)-5,6-bis(2-[5-furyl sulfonic acid])1,2,4-triazine sodium salt (ferrozine) > 1mmol/l ,detergents 0.12 %

PROCEDURE: These reagents may be used both for manual assay Sample Start method in automatic analyzer at 550nm at 37°C. Sample Start method -Mix well, incubate 10 min. at 37°C. Then read absorbance of test blank a (TB) against blank (B) and absorbance of test sample a (T) and standard sample a(S) against reagent blank (RB). The colour is stable for 30 min from sample addition. Calculate absorbance value of standard and test samples:

A (T) = a (T) – a(TB)A(S) = a(S) - a (TB)

Calculation

Ferrum concentration = A(T) /A(S) x standard / calibrator concentration

DISCUSSION

After 12 weeks of treatment. There was significant difference in the increase in serum iron taking the probiotic LP299V compared with controls {Control: 80.6mg/dl to 79.99mg/dl, Diet: 50.05mg/dl to 51.1mg/dl, Folic acid+Diet: 45.15mg/dl to 48.35mg/dl, Probiotic+Folic acid+Diet: 64.6mg/dl to 74.1mg/dl}. Additionally, an increase in iron level was significantly associated with probiotics and folic acid use when controlling for other factors including subjective weight. Overall, the treatments were well tolerated, with mild side effects.

CONCLUSION

Only about 10-15% of the iron ingested through the normal diet is absorbed by the body. In addition to a relatively low natural absorption of iron from the diet iron loss occur from the body through bleeding during menstrual periods. This may have big impact on iron levels leading to anemia common in

women of child bearing age. Iron absorption concept based on LP229V in combination with iron and folic acid is clinically proven to increase iron uptake. The most efficient way of iron absorption is by consuming iron rich food with vitamin c, along with folic acid and a probiotic LP299V.

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