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Research Article

Supplementation of Phytase Producing Probiotic *Pediococcus acidilactici* BNS5B Ameliorates the Bioavailability of Iron in Female BALB/c Mice Fed with Phytic Acid Rich Diet

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Abstract

Nutritional iron deficiency anemia occurs either due to low iron intake or low bioavailability of iron in vegetarian diet rich in phytic-acid, the anti-nutritional factor. Phytic acid causes iron deficiency due to the formation of non-degradable iron-phytic acid complex under alkaline pH of gastrointestinal tract. Therefore, the study was designed to assess the effect of supplementation of indigenous phytase producing probiotic *Pediococcus acidilactici* BNS5B vis-a-vis exogenously Phytase Modified Diet (PMD) in diet induced murine anemia. Female BALB/c mice were divided in five groups. Three groups were fed with either Standard Pellet Diet (SPD), Phytic Acid Rich Diet (PARD) or PMD and two groups were regularly supplemented with probiotic *P. acidilactici* BNS5B with PARD (PARD+P) and SPD (SPD+P) diet. Hemoglobin, Lactic Acid Bacterial count (LAB), serum ferritin, serum, liver and spleen iron concentration were monitored at an interval of 9 post diet feeding for a period of 45 day with histological and iron grading of spleen and liver. It was found that regular supplementation of *P. acidilactici* BNS5B or PMD alone led to significant increase ($p<0.05$) in haemoglobin, serum ferritin concentration, iron concentration in both serum and tissue (spleen and liver) of SPD+P, PARD+P and PMD compared with SPD and PARD animals. More specifically, the histology and iron status of both liver and spleen was also restored. It can be concluded that regular supplementation of *P. acidilactici* BNS5B could be used as an effective alternate bio fortification strategy in replenishing the iron that in turn may attenuate the incidence of diet induced anemia.

Keywords: Iron Deficiency Anemia; Phytic Acid; Phytase; Probiotic; Bioavailability

Abbreviations

IDA: Iron Deficiency Anemia; NFHS: National Family Health Survey; SPD: Standard pellet diet; PARD: Phytic Acid Rich Diet; IAEC: Institutional Animal Ethical Committee; PMD: Phytase Modified Diet; LAB: Lactic Acid Bacteria; H&E: Hematoxylin and Eosin; CFU: Colony Forming Units; FTU: Phytase activity Unit; GIT: Gastrointestinal Tract; ICP-MS: Inductively Coupled Plasma-Mass Spectrometry.

Introduction

Iron, an essential micronutrient involved in various physiological processes such as oxygen transport, oxidative metabolism, cellular proliferation etc and its deficiency leads to life threatening conditions ranging from anemia to neurological disorder. In diet iron is available either as easily absorbable heme iron or non-absorbable non-heme iron where the former is derived from meat product and latter is derived from plant based foods [1,2]. Though, etiology of iron deficiency is multifactorial, but major factors seems to be either low intake of iron or reduced bioavailability due to poor dietary diversity, poor nutrient density of staple-based complementary foods and presence of anti-nutritional factors such as polyphenols, oxalic acid and phytic acid in plant based diet [3,4]. Vegetarian diet is plagued by low iron content and poor absorption mainly due to phytic acid leading to iron deficiency [5]. Iron deficiency with or without anemia affects millions of lives mainly women and children in both underdeveloped and developing countries [6]. Global Burden of Disease Study, rated Iron-Deficiency Anaemia (IDA) among the top 10 causes of disability-adjusted life years for women [7,8]. National Family Health Survey (NFHS-4) carried out by the Ministry of Health and Family Welfare reported the prevalence of anaemia as 58.6, 53.1, 50.4 and 22.7 per cent respectively, among children aged 6-59 months, women aged 15-49y, pregnant women aged 15-49y and men aged 15-49y [9].

Several strategies have been adapted to reduce the prevalence of nutritional anemia such as iron supplementation *via* tablets, syrups, fortification of staple food with effective iron compounds, dietary modification and diversification [10,11]. More specifically, it has been observed that iron deficiency occurring due to anti-nutritional factors present in diet is not even resolved by adopting iron-rich diet as absorption of iron is very much dependent on the amount of phytic

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acid [12].

Phytic acid, the main phosphate storage component of plant based diet, is one of the prime causes of iron deficiency due to its ability to chelate iron thus reducing the bioavailability [13]. To enhance the iron availability, phytate content in food needs to be decreased either by chemical or biological processes such as soaking, milling, grinding, fermentation, activation of endogenous plant phytases or by application of exogenous microbial phytase [14]. However, the extent to which phytic acid can be reduced by chemical/ biological processes vary greatly with the processing method and require optimum conditions that results in inactivation of the endogenous thermo-labile plant phytases. Thus, the complete degradation of phytic acid can be achieved only by the action of exogenous microbial phytase that catalyzes the hydrolysis of phosphate group releasing the various chelated minerals. It has also been reported that addition of live beneficial lactic acid bacteria which when administered (probiotics) adequately in diet degrades the unknown inhibitors that has also been found to be beneficial in iron absorption [15]. Probiotics have multi-factorial benefits and has been recommended even by WHO to be used either as the prophylactic and therapeutic biointerventions for both the nutritional and health benefits [16,17]. Interestingly, we have isolated Lactic Acid Bacteria from neonates feces that has been found to possess all the probiotic attributes and produce phytase enzyme [18]. Combining the nutritional and growth benefits of phytases and probiotic, it is hypothesized that probiotic with phytase potential can be employed to degrade phytate in gastrointestinal tract thereby enhancing the bioavailability of iron and attenuating the anemia due to iron deficiency. Keeping these information in mind, it seems both experimentally and clinically non much information is available pertaining to application of probiotic in ameliorating the bioavailability of micronutrients and warrants further investigations. Thus, it was pertinent to assess the effect of supplementation of phytase producing probiotic *Pediococcus acidilactici* BNS5B vis-à-vis its enzyme phytase in ameliorating the bioavailability of iron in murine model.

Materials and Methods

Probiotic strain

A well characterised probiotic *Pediococcus acidilactici* BNS5B was grown in de Man Rogosa Sharpe broth by incubating at 37°C for 24 h, cold centrifuged at 7836 g for 10min, washed and the cells were suspended in sterile sodium acetate- acetic acid buffer (0.1M, pH 5.5) to contain 1×10^{10} CFU/mL for oral administration [18].

Preparation of Phytic Acid Rich Diet (PARD)

Phytic acid rich diet (PARD) was prepared as per phytic acid content of the staple grains mainly whole wheat, rice, pearl millet, chick peas, pinto beans, black eyes beans, soyabean etc consumed by vegetarian population. Flours of wheat, soybean, pinto beans, pearl millet and wheat bran were mixed in 3:1:3:1:1 ratio having carbohydrate, fat and protein similar to Standard Pellet Diet (SPD) procured from Ashirwad, Ropar, India (Table 1). The flours were mixed, dough was kneaded, pellets were prepared and baked at 100°C for 30 to 45 min [19].

Table 1: Composition of Standard Pellet Diet (SPD) and Phytic Acid Rich Diet (PARD).

S.No.	Ingredients	SPD (%)	PARD (%)
1	Carbohydrates	65.32	52.96
2	Crude protein	18	17.12
3	Crude fat	4.98	3.93
4	Crude fiber	3.28	19.56
5	Energy	3.04	3.57

Monitoring of enzyme concentration and preparation of Phytase Modified Diet (PMD)

In order to assess the amount of phytase required in phytase modified diet, PARD was treated with different concentrations of phytase (0-350 FTU/Kg) obtained from probiotic *P. acidilactici* BNS5B. The minimum concentration of exogenous phytase which showed maximum dephytinisation releasing phosphorous was selected and phytase modified diet was prepared [20]. Thereafter, PARD flour mixture was kneaded with appropriate amount of phytase, incubated overnight at 37°C, pellets were prepared and baked as described for PARD preparation [19].

Animals

Female BALB/c mice (20-25g) were housed in polypropylene cages in the animal house and were acclimatized for 7–10 days under the standard conditions of light and dark cycle before being used and were provided required diet and water ad libtium. Care, use and disposal of animals was done in accordance with the guidelines of the Institutional Animals Ethical Committee (IAEC), Chandigarh and approved by the Committee for the Purpose of Control and Supervision on Experiments on Animals (PU/IAEC/S/15/83).

Study design

210 animals were divided in 5 groups each containing 42 mice; Group I (SPD): These animals were fed with standard pellet diet for 45days; Group II (SPD + *P. acidilactici* BNS5B (SPD+P): Animals belonging to this group were fed orally daily with a single dose of probiotic (1×10^{10} CFU/mL) and provided SPD for 45 days; Group III (PARD): These animals were fed with PARD for 45 days; Group IV (PARD + *P. acidilactici* BNS5B (PARD+P)): These animals were fed orally daily with probiotic (1×10^{10} CFU/mL) and provided PARD for 45 days; Group V (PMD): Animals belonging to this group were fed with PMD for 45 days.

Follow up of animals

After respective treatment of animals belonging to different groups, body mass, hemoglobin concentration, phosphorus, phytic acid and lactic acid bacteria in feces were assessed at a regular interval of 9 days up to 45 days. Additionally, blood was collected by retro-orbital bleed route from 6 mice belonging to each group and thereafter, these were sacrificed by cervical dislocation and both liver and spleen were removed for histological studies.

Estimation of body mass

The body mass of mice belonging to each group was determined using digital balance, Chandigarh, India) at an interval of 9 days up to 45 days.

Hemoglobin level

Hemoglobin (Hb) level was calculated calorimetrically in blood at an interval of 9 days up to 45 days using standard Hb solution [21].

Table 2: Categories of iron deficiency anemia based on hemoglobin concentration (g/dL) for female mice.

Anemia category	Hemoglobin level (g/dl)
Normal	≥ 13.9
Very mild	12.8-13.8
Mild	11.0-12.7
Moderate	9.3-10.9
Severe	7.5-9.2

Briefly, 20 µL of whole-blood was added to 5ml Drabkin's reagent (Sigma, India), kept for 15 min and optical density was measured at 540 nm. The amount of Hb was expressed as g/dL and was calculated as

$$C(g/dl) = \frac{\text{OD of test}}{\text{OD of standard}} \times \text{Concentration of standard}$$

On the basis of Hb, animals were categorized as per Raabe et al. [22] (Table 2).

Enumeration of lactic acid bacteria count in feces

In order to assess the Lactic Acid Bacteria in the intestine, freshly voided fecal material (0.1g/mouse) from each group was homogenized in phosphate buffer saline and serially diluted at an interval of 9 days up to 45 days. The diluted homogenates (0.1ml) were spread plated on MRS agar plates and incubated at 37°C for 24 hours and results were interpreted as Colony Forming Units (CFU/ml; 23).

Assessment of free phosphorus and phytic acid content in feces

Free phosphorus and phytic acid were assessed done at a regular interval of 9 days up to 45 days using total phosphorous and phytic acid K-PHYT kit (Megazyme International, Bray, Country Wicklow, Ireland). Briefly, 1.0g of fecal sample was collected, homogenized in 20mL HCl (0.66M) for 3h at room temperature, microcentrifuged at 13,226 g for 10 minutes and supernatant was neutralized with NaOH (0.5M) in 1:1 ratio. 900µl of neutralized sample was treated with phytase as per manufacturer's instruction. Thereafter, 1 ml of coloring reagent [freshly prepared by mixing 4 volumes of ammonium molybdate (2.5% w/v) in sulphuric acid solution (5.5% v/v) and 1 volume of ferrous sulfate (2.5% w/v)] was added and the released inorganic phosphate was measured spectrophotometrically at 700 nm [24]. The amount of molybdenum blue formed during the reaction is directly proportional to the amount of inorganic phosphorus present in the sample and free phosphorous and phytic acid content was expressed as g/100g.

Serum preparation

Blood was withdrawn from mice before dissection by retro-orbital route in sterile test tubes without anticoagulant, kept at room temp for 2h and refrigerated overnight. Thereafter, the samples were centrifuged at 704 g for 10min and serum separated was collected carefully in clean eppendorf tubes for further use.

Serum ferritin assay

Serum ferritin was determined using The Mouse FE (Ferritin) ELISA Kit (Elabscience, USA) and assay was performed as per the manufacturer's protocol. Briefly, 100µL of appropriately diluted serum was added to each well of microtitre ELISA plate and incubated at 37°C for 90 minutes. Thereafter, the sample was removed and 100µL of biotinylated detection antibody was added, incubated at 37°C for 1 hour and washed 3 times with washing buffer. Thereafter, 100µL of Avidin-HRP conjugate was added, incubated for 30 minutes at 37°C, washed 5 times with washing buffer followed by the addition of 90µL of substrate reagent to each well and incubated for 15 minutes at 37°C. Finally, the reaction was stopped by addition of 50µL of stop solution to each well and optical density was measured at 450nm using ELISA reader (Biorad, USA) and results were expressed as ng/mL.

Determination of iron concentration

Iron concentration was determined in serum, spleen and liver homogenate by employing Inductively Coupled Plasma-Mass Spectrometry (ICP-MS: 77006, Agilent technologies, Snata Clara, CA) following microwave digestion. Tissue (liver, spleen) samples were weighed and homogenized (1:10 w/v) in 0.15M NaCl in 10mM NaOH-hepes buffer (pH 7.0) using a 1ml glass dounce homogenizer (Wheaton Scientific, Millville, N.J., USA). Briefly, appropriately diluted sample (serum, both spleen and liver homogenate) was added to 10 ml of a concentrated acid mixture containing nitric acid and perchloric acid (HNO_3/$HClO_4$) mixed at a 4:1 v/v ratio and kept in microwave tubes, followed by heating at 120°C until digested sample reached to 1.0 ml. Thereafter, the tubes were cooled to room temperature and volume was made to 10 ml with deionized water. The iron concentration was analyzed using ICP-MS and values were expressed as mg Fe/g of organ weight [25].

Histopathological study

After the animals were sacrificed, liver and spleen were removed, fixed in 10% buffered formalin, processed, stained (H&E stain), dried, mounted and were observed under light microscope.

Iron grading

Iron deposition was estimated as per Perls's prussian blue reaction, liver and spleen were fixed in 10% buffered formalin for 36 hours, processed, and embedded in paraffin. Sections were cut at 5 micron and stained by Prussian blue stain employing freshly prepared acid ferrocyanide solution for about 30 min and thereafter with 0.5% aqueous neutral red for 30 seconds. A qualitative estimation of iron stores based on the visible haemosiderin and ferritin deposits was made according to an arbitrary scale, increasing in intensity from 0 to 6 [26].

Statistical analysis

Results were statistically analyzed and expressed as mean ± Standard Deviation (SD) of five to six experimental replicates. The inter group variation was Assessed by One Way Analysis of Variance (ANOVA) followed by Turkey's Multiple comparison done by using prism software. The statistical significance was defined as p and calculated at $p < 0.05$.

Results

Enzyme concentration for preparation of phytase modified diet

It was found that on varying the enzyme concentration from 0 to 350 FTU/Kg, the amount of phytic acid decreased from 7.11g/100g to 3.8g/100g and increased phosphorous concentration but maximum dephytinisation of PARD occurred with phytase at a concentration of 175 FTU/Kg as was evident by the maximum release of phosphorous

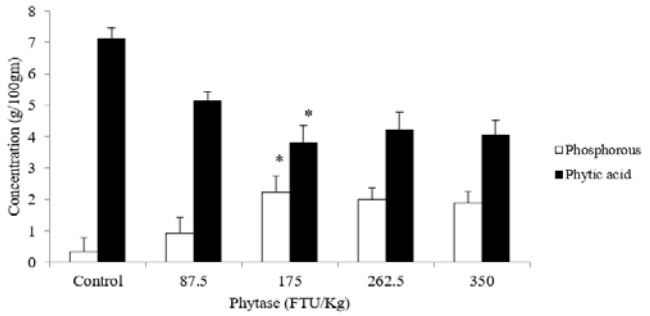

Figure 1: Effect of different concentration of exogenous phytase from *P. acidilactici* BNS5B on dephytinisation of PARD. Values are expressed as Mean±SD, *p<0.05 *vs* Control.

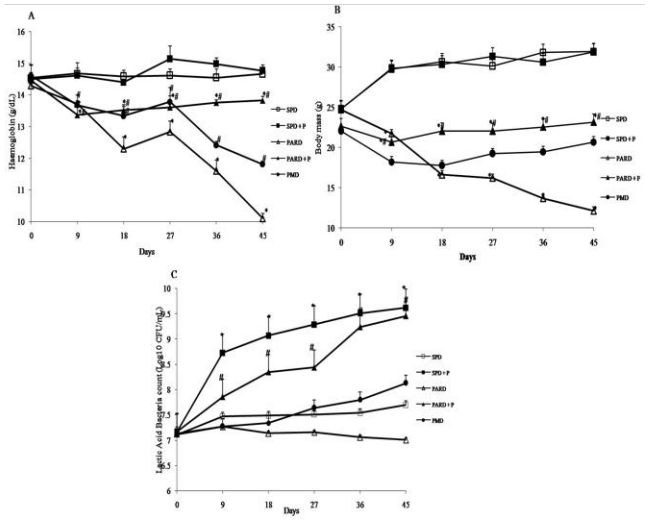

Figure 2: A) Haemoglobin (g/dL), B) Body mass (g) and C) Fecal Lactic Acid Bacteria count (log_{10}CFU/mL) of animals belonging to different groups at different days. Values are expressed as mean±SD, *p<0.05 *vs* SPD, #p<0.05 *vs* PARD.

and was used to prepare the Phytase Modified Diet that was fed to PMD (Group V) animals (Figure 1).

Haemoglobin concentration

Initially, Hb an important index of iron deficiency and anemia was found to be in the normal range (>14.3±0.2 g/dL) for animals belonging to all the groups (Group I, II, III, IV, V) and remained unaltered till the end of experiment in animals belonging to SPD (Group I) and SPD+P (Group II). However, Hb levels gradually decreased significantly (p<0.05) in PARD (Group III) animals and were categorised as moderately anemic. Interestingly, the Hb levels improved significantly (p<0.05) in animals belonging to PARD+P (Group IV, 13.83±0.26g/dL) and PMD (Group V, 11.81±0.65g/dL) at the end of experiment compared with PARD (Group II, 10.1±0.15g/dL) and were labelled as very mildly anemic and mildly anemic respectively (Figure 2A).

Body mass

Feeding of animals with PARD (Group III) decreased the body mass of animals significantly (p<0.05) at each point of observation compared with SPD (Group 1) and SPD+P (Group II) animals where

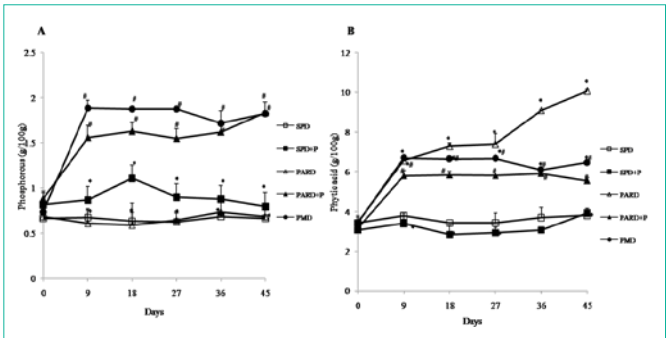

Figure 3: Concentration of A) Phosphorous (g/100g) and B) Phytic acid (g/100g) in the feces of animals belonging to different groups at different days. Values are expressed as mean±SD, *p<0.05 *vs* SPD, #p<0.05 *vs* PARD.

body mass was almost similar with minor difference from day 20 onwards. It was interesting to observe, that feeding animals either with probiotic *P. acidilactici* BNS5B to PARD (Group IV) or PMD (Group V) led to significant (p<0.05) increase in the body mass from beginning of experiment till day 45 compared with PARD (Group III) animals (Figure 2B).

Fecal Lactic Acid Bacteria (LAB) count

It was found that feeding of animals either with SPD (Group I) or PARD (Group III) had almost similar LAB count throughout the study but supplementation of probiotic *P. acidilactici* BNS5B led to significant (p<0.05) increase in fecal LAB count of animals belonging to SPD+ P (Group II) and PARD+P (Group IV) at each point of observation and only with minor increase in the LAB count of animals belonging to PMD (Group V) compared with SPD (Group I) and PARD (Group III) animals respectively (Figure 2C).

Fecal phosphorous and phytic acid

It was observed that animals fed with PARD (Group III) had not much evident effect of phosphorous excretion but had significantly (p<0.05) higher phytic acid in feces compared with SPD (Group I) animals at each point of observation. Interestingly, the daily oral supplementation of probiotic *P. acidilactici* BNS5B to PARD+P (Group IV) and SPD+P (Group II) animals led to significantly (p<0.05) increased level of fecal phosphorus and decreased phytic acid compared with PARD (Group III) and SPD (Group I) respectively. Though, animals fed with PMD (Group V) also showed significantly (p<0.05) decreased phytic acid and increased phosphorous excretion compared with PARD (Group III) but the change was less compared with PARD+ P (Group IV) animals (Figure 3A and 3B).

Serum ferritin concentration

It was observed that regular feeding of PARD (Group III) to animals for 45 days led to significant (p<0.05) gradual decrease in serum ferritin concentration (10.05±1.65 ng/mL) compared with SPD (Group I, 254.17±1.32 ng/mL). Interestingly, the regular supplementation of probiotic *P. acidilactici* BNS5B to animals belonging to both SPD (Group II) and PARD (Group IV) significantly (p<0.05) increased the serum ferritin concentration (265.75±3.53 ng/mL and 237.5±3.03 ng/mL respectively) at the end of study. Further, it was also observed that feeding of animals with PMD (Group V) also lead to significant (p<0.05) increase in the serum ferritin concentration (195.83±2.27 ng/mL) compared with PARD (Group

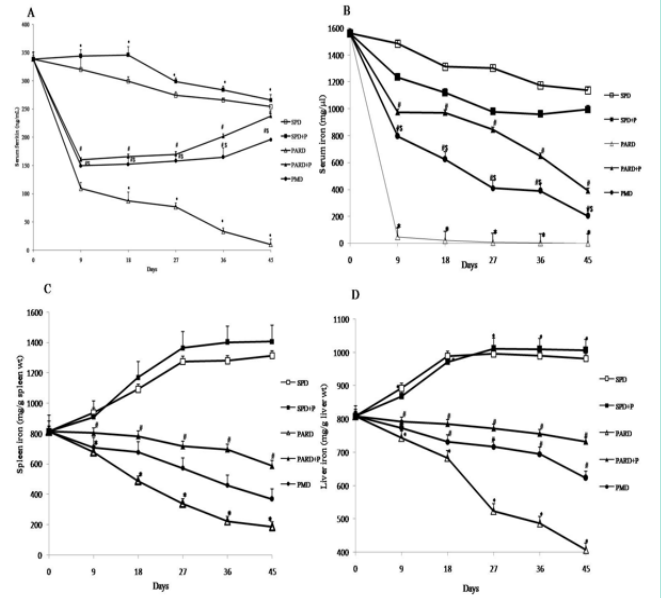

Figure 4: A) Serum ferritin levels (ng/mL), B) Serum iron concentration (mg/µl), C) Spleen iron concentration (mg/g spleen weight) and D) Liver iron concentration (mg/g liver weight) in animals belonging to different groups at different days. Values are expressed as mean±SD *p<0.05 *vs* SPD, #p<0.05 *vs* PARD, $p<0.05 *vs* PARD+P.

III) but was significantly (p<0.05) less compared with PARD+P animals (Figure 4A).

Serum iron concentration

Serum iron concentration (0.95±11.23mg/µL) was significantly (p<0.05) reduced in PARD (Group III) animals over the period of time compared with animals belonging to SPD (Group I, 1136.92±54.87 mg/µL) but daily supplementation of probiotic reduced the concentration of serum iron (995.36 ± 28.54 mg/µL) in SPD+P animals. Though, daily supplementation of either probiotic or phytase modified diet to animals belonging to PARD+P (Group IV) or PMD (Group V) led to significant (p<0.05) increase in serum iron concentration (392.15±13.53 mg/µL and 202.86±17.67mg/µL) compared with PARD (Group III) animals but the increase was less significant (p<0.05) in PMD (Group V) compared with PARD+P (Group IV) animals (Figure 4B).

Spleen and liver iron

It was found that the regular administration of PARD (Group III) to animals led to significant (p<0.05) decrease in the level of iron gradually in systemic reserves i.e. spleen (185.8±4.57 mg/g) and liver (406.91±34.58 mg/g) compared with SPD (Group I, 1313.88±2.21 mg/g spleen wt and 981.77±12.65 mg/g liver wt) by the end of study. Interestingly, the regular supplementation of probiotic *P. acidilactici* BNS5B to animals belonging to PARD+P (Group IV) showed significant (p<0.05) increase in iron concentration in both spleen (587±6.13 mg/g) and liver (732.12±13.76 mg/g) compared with PARD animals (Group III) by the end of study. Though, the concentration of iron was found to increase gradually over the period of time in spleen (368.56±3.74 mg/g) and liver (622.94±32.7 mg/g) of animals belonging to PMD (Group V) but was significantly (p<0.05) less compared with PARD+P (Group IV) animals (Figure 4C and 4D).

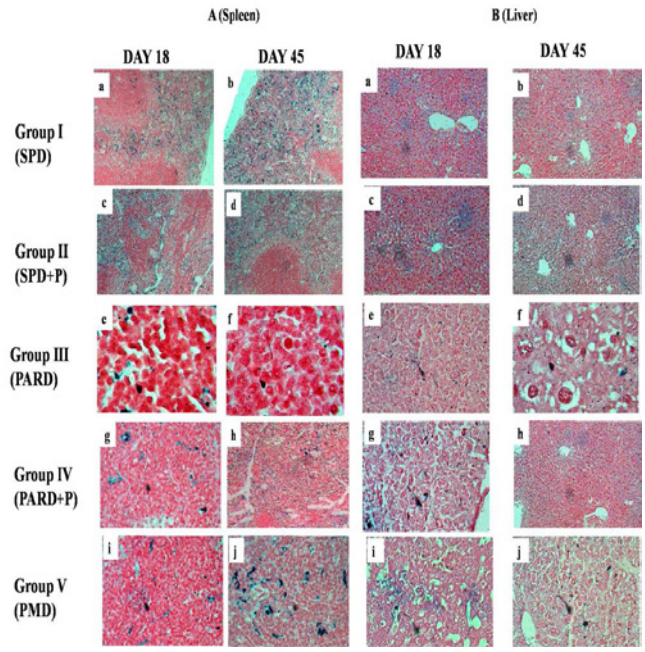

Figure 5: Pearl's prussian stained photomicrograph of A) Spleen of animals belonging to various groups on day 18 (a, c, e, g, i) and 45 (b, d, f, h, j) showing: iron deficiency of Grade VI (100X) in SPD and SPD+P animals (a, b, c, d); Grade II (1000X) in PARD (e); Grade I (1000X) in PARD (f); Grade IV (450X) in PARD+P (g); Grade V (100X) in PARD+P (h); Grade IV (450X) in PMD (i); Grade III (450X) in PMD (j) and B) Liver of animals belonging to various groups on day 18 (a, c, e, g, i) day 45 (b, d, f, h, j) showing: iron deficiency of Grade VI (100X) in SPD and SPD+P animals (a, b, c, d); Grade III (450X) in PARD (e), Grade I (1000X) in PARD (f); Grade IV (450X) in PARD+P (g); Grade V (100X) in PARD+P (h); Grade IV (450X) in PMD (i); Grade III (450X) in PMD (j).

Histopathological and iron store grading study

Perl's stained slides (Prussian blue reaction) demonstrates the amount of iron stores as indicated by presence of haemosiderin (blue condensed granules) and ferritin (blue cytoplasmic blush) in both the tissues i.e. spleen and liver. It was observed that the spleen of PARD (Group III) fed animals showed retrogression in haemosiderin and ferritin on both day 18 and 45 indicating Grade II and Grade I iron deficiency compared with Grade VI of SPD (Group I) and SPD+P (Group II) animals (Figure 5(A): a, b, c, d, e and f). Further, it was interesting to observe that haemosiderin and ferritin granules increased progressively with daily supplementation of probiotic *P. acidilactici* BNS5B to animals belonging to PARD+P (Group IV) that had Grade IV and V on day 18 and 45 respectively (Figure 5(A): g and h). Although, the iron stores also increased in spleen of PMD (Group V) animals and had Grade IV and III on day 18 and 45 but the restoration of iron reserves was less compared with PARD+P (Group IV) animals (Figure 5(A): i and j).

Similarly, Perl's stained liver also showed that PARD (Group III) animals had decreased haemosiderin and ferritin with Grade III and Grade I iron deficiency on day 18 and 45 compared with Grade VI of SPD (Group I) animals (Figure 5(B): a, b, c, d, e and f). Interestingly, both probiotic and phytase modified diet supplementation to animals for 45 days showed enhanced iron stores of Grade IV and Grade V

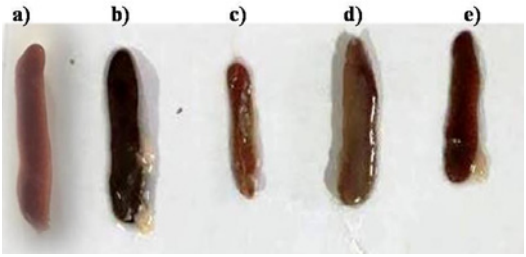

Figure 6: Gross observation of spleen of animals belonging to various groups on day 45: (a) SPD; (b) SPD+P; (c) PARD; (d) PARD+P; (e) PMD.

in PARD+P (Group IV) and Grade IV and III in PMD (Group V) animals on day 18 and 45 respectively (Figure 5(B): g, h, i and j).

Macroscopically, it was interesting to observe that the spleen of animals belonging to PARD (Group III) and PMD (Group V) was smaller in size compared with SPD (Group I), SPD+P (Group II), PARD+P (Group V) animals (Figure 6). Histopathologically, the spleen and liver section of SPD (Group I) and SPD+P (Group II) animals showed normal splenic follicles with distinct red and white pulp, radially arranged liver cells in single rows from central venules, normal hepatocytes and hepatic sinusoids compared with no distinct red pulp and white pulp in spleen and disoriented liver cells with slightly expanded hepatic sinusoids accompanied by fat vacuoles in hepatocytes of PARD (Group III) animals (Figure 7(A): a, b, c, d, e and f; Figure 7(B): a, b, c, d, e and f). Interestingly, both probiotic and phytase modified diet supplementation to SPD (Group II), PARD (Group IV) and PMD (Group V) animals for 45 days showed almost normal histology of both spleen and liver compared with SPD (Group I) animals (Figure 7(A): g, h, i and j; Figure 7(B): g, h, i and j).

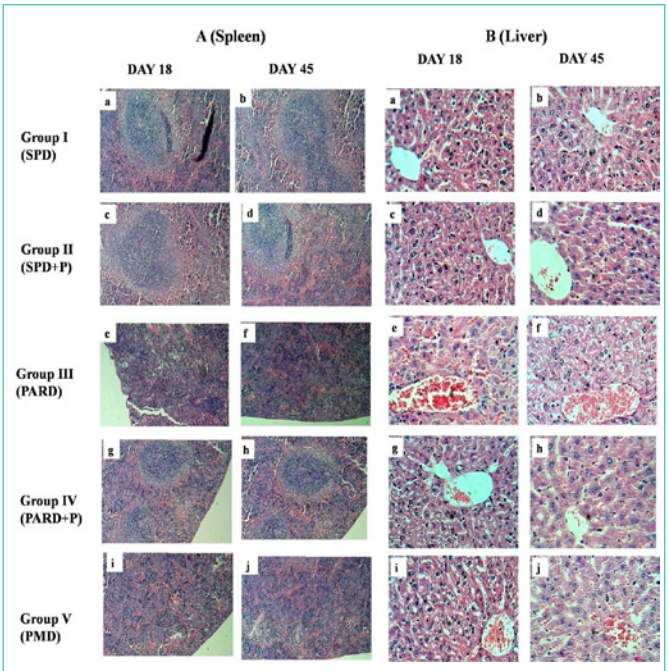

Figure 7: Histopathological alterations in H&E stained A) Spleen of animals (100X) belonging to various groups on day 18 (a, c, e, g, i) and 45 (b, d, f, h, j) showing: normal splenic follicles in SPD and SPD+P animals (a, b, c, d); no distinct red and white pulp with absence of splenic follicles in PARD (e, f) and PMD (i, j); smaller splenic follicles in PARD+P (g, h) and B) Liver of animals (400X) belonging to various groups on day 18 (a, c, e, g, i) and 45 (b, d, f, h, j) showing normal radially arranged hepatocytes and hepatic sinusoids in SPD and SPD+P animals (a, b, c, d); disorganized hepatocytes with abundant fat vacuoles and expanded hepatic sinusoids in PARD (e, f); radial arrangement of hepatocytes with decreased fat vacuoles and hepatic sinusoids narrowing towards normal histology in PARD+P (g, h) and PMD (i, j).

Discussion

Iron balance in body is regulated by dietary iron absorption and systemic factors as there is no means of excretion of iron from body. Dietary iron absorption is highly affected in vegetarians due to the presence of phytic acid in diet [11]. Current approaches to improve iron status in individuals are iron supplements or fortification of common food with iron as low iron concentration causes disorders such as anemia but high concentration of iron due to fortification or regular supplementation in food may lead to synthesis of free radicals of iron which in turn enhances the incidence of colon cancer [27]. Moreover, fortification strategies are not suitable for developing and under developed countries, as they rely on effective regulatory agencies, infrastructure, robust distribution, consumer awareness, and inability of poor to afford [28,29]. Thereby, the most sustainable and cost effective approach is 'biofortification', a process that improves the absorption of native iron present in foods either *via* consumption of phytase producing probiotic or phytase treatment that catalyzes food before consumption as a means of pretreatment or in the Gastrointestinal Tract (GIT) *via* phytase producing microorganism. Therefore, our aim was to ameliorate the bioavailability of iron either by supplementation of indigeneous probiotic *P. acidilactici* BNS5B due to its phytic acid catalytic action or pretreatment of PARD with exogeneous phytase being produced by *P. acidilactici* BNS5B in female BALB/c mice.

The experimental Phytic Acid Rich Diet (PARD) was prepared as per the staple diet of vegetarian population of India that had carbohydrate, fats, proteins and energy content similar to Standard Pellet Diet (SPD) with an exception of high content of fiber mainly comprising of phytic acid. Diet composition used in present study was very different from earlier studies where scientists have used either wheat, sorghum, corn, soybean or sesame alone for assessing the effect of such diet on the bioavailability of calcium, iron, zinc [30,31]. Further, it was found that 175 FTU/Kg of phytase was optimum for the pretreatment of PARD as it enhanced *in vitro* dephytinisation i.e. enhancing the phosphorous availability and decreasing the phytic acid. More specifically, it was observed that with increased concentration of phytase, not much change in dephytinisation of phytic acid was observed as phosphorous release was constant and corroborated with earlier studies [32,33]. These scientists have also observed that on increasing the phosphate concentration in diets, the phytase synthesis decreased due to catabolic repression of phytase in the presence of orthophosphate at transcriptional level.

It was observed that feeding of PARD for a period of 45 days resulted in decreased Hb levels i.e. drifting the Hb from normal to moderately anemic and is in concordance with earlier study where animals fed with iron deficient diet had similar effect on Hb [34]. Interestingly, the enhanced Hb levels in animals fed with probiotic along with PARD or PMD may be due to dephytinisation of phytic acid by phytase either produced by probiotic *P. acidilactici* BNS5B or pretreatment of diet. Earlier studies have also reported that phytases

mediate the release of iron from cereal based food that in turn affect the availability of iron in poultry, pigs and humans [35-37].

Normally, phytic acid exists in a complex form with phosphorus, an important micronutrient required for the body mass and renders it unavailable for the absorption resulting in decreased bone density eventually causing decrease in the body weight and may be one of the reason for observed decrease in body mass of PARD animals. Adeola and Cowieson, [38] have also demonstrated that non ruminant animals fed with plant originated feed may limit the phosphorous utilization hence their diets are often supplemented with the inorganic phosphorus so as to fulfill the phosphorous requirements. Interestingly, we found that supplementation of diet with phytase produced by probiotic or as exogenous treatment, increased body mass occuring due to dietary phytase that dephytinized phytic acid as was evident by reduced phytic acid and increased phosphorous excretion in feces [39,40]. These studies have also observed that addition of wheat endogenous phytase almost completely degraded phytic acid from fonio porridge and enhanced phosphorous level. Moreover, other scientists have also observed weight gains in broilers, chicks, catfish, broiler, rainbow trout and labeo rohita fed with recombinant phytase producing Lactobacillus gasseri TDCC65 or either with plant, microbial or commercial phytase [30,41,42].

LAB are known to have beneficial effect on host by modifying gut microbiota, microenvironment and ameliorating immune response due to production of various metabolites. In present study, we also observed that daily supplementation of probiotic *P. acidilactici* BNS5B either with PARD or SPD, enhanced fecal LAB count and is in accordance with earlier studies [23,43,44]. However, increased fecal LAB count in PMD animals may be due to generation of phosphorylated compounds on pretreatment of PARD with phytase that may have been metabolized by the gut microbiota, thus restoring the bacterial eubiosis in the gastrointestinal tract [45,56].

Serum iron is an indicator of available iron whereas serum ferritin concentration reflects the iron stores in various tissues [47]. Interestingly, increased level of serum iron in animals supplemented with phytase producing probiotic *P. acidilacticici* BNS5B or phytase pretreated PMD may be due to the anti-chelating nature of phytase on non-haeme phytate bound iron and corroborates with earlier studies [35,48]. These researchers have also observed that addition of phytase from *Aspergillus niger* to cereal porridge enhanced the iron absorption from 14 to 26%. Similarly, serum ferritin level increased in both liver and spleen with both probiotic supplementation and phytase modified diet mainly due to replenishment of iron stores in spleen and liver as is supported by enhanced level of iron concentration in both spleen and liver homogenates. This may be due to the increased bioavailability of food iron by the action of phytase on phytic acid due to probiotic or by exogenous phytase [49]. Several scientists have also reported increased hepatic and serum ferritin levels in humans, pigs and poultry when fed with vegetable food crop *Telfairia occidentalis, Aspergillus niger* fungus phytase, *Escherichia coli* phytase and commercially available phytases [21,50].

In order to assess the iron deficiency occurring due to diet, iron stored in spleen and liver were also monitored as deposits of iron in tissues is stored as haemosiderin (stable form, less mobilizable) and ferritin (readily mobilizable) forms [51-53]. Therefore, stained haemosiderin and ferritin were employed to grade the liver and spleen sections with reference to iron deficiency [26]. It was interesting to observe that animals fed with PARD had gradually decreased haemosiderin and ferritin both in liver and spleen indicating iron deficiency and is also supported by the altered liver histology occurring either due to inhibition of DNA synthesis in liver due to iron deficiency anemia and disrupted lipid metabolism and disappearance of splenic follicles in spleen [54-56]. Interestingly, the daily supplementation of either probiotic or PMD positively synchronized the iron stores by replenishing both haemosiderin and ferritin along with normal histology of liver and is in accordance with earlier studies where scientists have reported that iron fortification to iron deficient rats and the administration of *A. niger phytase* fortified cereals increased iron in liver thereby repairing tissue histology [56-58].

Conclusion

Taken together, this is the first ever such experimental study suggesting that either regular supplementation of phytase producing probiotic *P. acidilactici* BNS5B or pretreatment of diet with phytase from probiotic could be used as an alternative biofortification strategy to ameliorating iron deficiency anemia by modifying the metabolism and physiology leading to improved haemoglobin, serum iron, serum ferritin levels and replenishing the iron stores in the vegetarian population. Further, it is reasonably suggested that probiotic *P. acidilactici* BNS5B may not only be employed for ameliorating iron deficiency anemia but may also be used to combat other nutritional deficiencies too and needs to be validated clinically.

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