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Effectiveness of Functional Wheat - Fermented Milk Beverage against Tannic Acid Induced Anemia.

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ABSTRACT

Aiming to evaluate the effectiveness of functional cereal-fermented milk beverage against anemia in rats. The experiment was carried out by dividing rats into three groups. Negative control (normal) was fed on standard diet, positive control group was fed on standard diet + tannic acid (15 gm / kg diet) and the third group was fed on standard diet + tannic acid (15 gm /kg diet) + 20% of tested functional beverage. Our results revealed that the probiotic containing beverage ameliorated blood hemoglobin, serum iron, total iron binding capacity, ferritin, lipid peroxidation and total antioxidant capacity. Histopathological examination of the anemic rats showed disturbed splenic pulps with congested blood vessels and lymphocytic infiltration around the central vein. These changes were markedly restored by the tested functional beverage. It was concluded that foods containing folic acid and riboflavin - producing encapsulated probiotic bacteria enhanced iron absorption and utilization. Hence it protects against iron deficiency anemia.

Keywords: Fermented Beverage, Riboflavin, Folate, *Streptococcus thermophilus*, Microencapsulation, Anemia, Tannic acid, Oxidative stress.

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Introduction

Anemia is one of the world's most prevalent public health problems affecting >1.6 billion people world wide. It is associated with serious health problems especially in children and pregnant women .Although the most common cause of nutritional anemia is iron deficiency, other possible causes include deficiencies of vitamin A, B12, B6, C, riboflavin and folic acid.

The micronutrients are known to affect the synthesis of hemoglobin (Hb) either directly or indirectly by affecting the absorption of iron (1).

Riboflavin (vitamin B2) deficiency is one of the most common vitamin deficiencies in developing countries. The inadequate riboflavin intake is an important risk factor for anemia (2). Also dietary folate deficiency is the leading cause of megaloblastic anemia in the world (3).

Probiotics are live microorganisms which when administered in adequate amounts confere a health benefit on the host (4). The beneficial effects attributed to probiotic including improvement of intestinal health, enhancement of the immune response, reduction of serum cholesterol and cancer prevention.

In fact there is substantial evidence to support probiotic use in the treatment of acute diarrheal diseases, prevention of antibiotic-associated diarrhea, and improvement of lactase metabolism (5). Furthermore the production of vitamins has been claimed among the causal relationships of the healthy benefits probiotics.

Probiotic bacteria mostly belonging to the genera *Lactobacillus* and *Bifidobacterium* confer a number of health benefits to the host including vitamin production (6 , 7).

The aim of the present study was to evaluate the biological effect of functional wheat - fermented milk beverage containing riboflavin and folic acid –producing encapsulated *Streptococcus thermophilus* on anemia in rats.

MATERIALS AND METHODS

The ingredients used for preparation of the diet given to the animals were purchased from the local market. These items were corn starch, sucrose, corn oil. Tannic acid was obtained from Elgomhorya Company for Chemicals and Drugs, Cairo; Egypt. Casein was obtained from Sisco Research Laboratories PVT.LTD India. Salts and vitamins used for the preparation of the salt and Vitamin mixtures were obtained from Merk, Germany and prepared according to (AI N95) (8).

Composition of tested wheat - fermented milk beverage:

This product previously made by the project team work at Dairy Microbiology Lab. National Research Center (Sharaf *et al.,* 2015) (9):

- Fresh high quality strawberry fruit (25%)
- Skimmed milk inoculated with encapsulated *Streptococcus thermophilus* (25%)
- Sterilized wheat extract (25%)
- Whey permeate (25%)

Besides to the tested functional beverage, standard normal diet were formulated and composed as shown in Table (1).

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Table (1): Composition of the standard and tested diet (g/100 gm)

Group / Ingredient	Group I -ve control	Group II +ve control	Group III tested diet
Casein	15	15	15
Corn oil	8	8	8
Sucrose	10	10	10
Cellulose	4	4	3
Deoxycellulose	-	-	1
Salt Mix	4	4	4
Vit Mix	1	1	1
Tannic acid	-	1.5	1.5
Tested formula	-	-	20
Starch	58	56.5	36.5
Total	100	100	100

Tested diet (Group III) enriched of microbial folic acid and riboflavin

The animals used in the biological experiment were Sprague Dawley rats obtained from the animal house of the National Research Center; the average body weight ranged between 120-130 gm and comprised both sexes.

Kits used for the estimation of the analyzed parameters were obtained from Biodiagnostic Company and Stanbio laboratories .

Design of Animal Experiment

The animal experiment comprised 3 groups each of 7 rats:-

- The 1st group was fed on standard diet and served as (negative control)
- The 2nd group was fed on standard diet + tannic acid (15 gm /kg diet) and served as (positive control) (10)
- The 3rd group was fed on standard diet + tannic acid (15 gm /kg diet) +20% tested functional beverage.

Animals were kept individually in stainless steel cages, deionized water was allowed ad-libitum to positive control and tested group. The room temperature was adjusted at 25°C. The feeding period continued for 6 weeks. During the experimental period the food consumption and body weight of the animals were followed.

The experimental procedure was carried out according to the Institutional Animals Ethics Community of the NRC, Egypt.

At the end of the experimental period (after 6 weeks) rats were fasted overnight. In the morning, the body weight was recorded. Non heparinized and heparinized blood samples were obtained by open heart puncture under slight diethyl ether anesthesia. Heparinized blood was used for estimation of hemoglobin concentration(Hb) and hematocrit percent (HCT). While serum was separated from non-heparinized blood samples by centrifugation at 3000r.p.m. for 10 min and then stored at -40 C until analysis of other parameters later.

Organs namely the liver, the kidney and the spleen were separated, washed with saline solution (0.9%) and blotted between two sheets of filter paper and then weighted and kept at - 20°C.

Biochemical analysis

Hemoglobin concentration was determined according to Betke and Savelsberg (1950) (11) while packed cell volume or hematocrit percent was determined according to AC.inory (1954) (12). Red blood cell count (R.B.Cs) was counted according to Fischbach (1996) (13). Serum samples were analyzed for estimation

of iron according to Bauer (1984)(14). Serum ferritin was determined according to the method described by Young (2000) (15), while total iron binding capacity (TIBC) in serum was measured according to Buritis and Edward (1994) (16). Concerning transferrin saturation (T sat) in serum, it was calculated as [serum iron/ serum TIBC (x100)].

On other hand serum malondialdehyde (MDA) was determined accoding to Satoh (1978) (17), total antioxidant capacity (TAC) was estimated according to Koracevic *et al.,* (2001) (18).

Histopathological examination

Spleen specimens from all animals were dissected immediately after death, and fixed in 10% neutral-buffered formal saline for at least 72 hours. All the specimens were washed in tap water for half an hour and then dehydrated in ascending grades of alcohol, cleared in xylene and embedded in paraffin. Serial sections of 6 µm thick were cut and stained with haematoxylin and eosin for histopathological investigation. Images were captured and processed using Adobe Photoshop version 8.0. (19).

Statistical analysis

Data are presented as mean ± SE. statistical analysis of the data were performed using SPSS-PC software. Unpaired student's t-test was used to compare biological differences. Meanwhile one way analysis of variance (ANOVA) was used for comparison of different biochemical values in various experimental groups. It was followed by Duncan's multiple range test to clarify the significance. P values less than 0.05 were considered significant.

RESULTS AND DISCUSSION

The use of vitamin-producing microorganisms may represent a more natural, more advantageous and consumer-friendly alternative to fortification using chemically synthesized pseudo-vitamins and would allow the production of foods with elevated concentrations of vitamins that are less likely to cause undesirable side-effects (20).

Hence, this study aimed to evaluate the biological effect of microbial production of riboflavin and folic on anemic rats.

Effect of experimented diets on body weight gain (BWG), food intake (FI), and feed efficiency ratio (FER) was illustrated in Table (2).

Table (2): Body weight gain, food intake and feed efficiency ratio of the different experimental groups

parameters / Treatments	Body Weight Gain (g)	Food intake (g)	Feed efficiency ratio
G-1 Negative Control	61.00 ±4.08a	420.00± 19.01b	0.146± 0.013a
G-2 Positive control	18.66± 6.75b	417.50 ± 10.30b	0.043 ± 0.016b
G-3 Tested group	74.50 ± 3.94a	474.16 ± 03.74a	0.156± 0.008a

All values represented as mean ±S.E.

Mean values at the same column sharing the same superscript letters are not significantly different (P<0.05)

The mean values of body weight gain of negative control, positive control and tested group were (61.0 ± 4.08, 18.66± 6.75, 74.50 ± 3.94 gm respectively), food intake were (420± 19.01, 417.50 ± 10.30 and 474.16 ± 3.74 gm respectively), while feed efficiency ratio were (0.146 ± 0.013, 0.043 ± 0.016 and 0.156± 0.008 respectively). It was found that the tested group recorded a significant increase in BWG, FI and FER compared with the positive control group (anemic group). It could be noticed that positive control group showed marked decrease in (BWG) and FER compared with healthy negative control group.

From the data, it can be noticed that rats fed on positive control containing tannic acid have a high food intake, but, feed efficiency ratio was very low and body weight gain was the lowest as compared to tested group or negative control.

On the other hand, BWG, FI and FER value of rats fed tested diet showed high significant increase as compared to those of positive control group. These results are in accordance with those of Deng &Wilson (2003)(21) and Li et al., (2010)(22), who reported that riboflavin significantly improved growth, feed efficiency ratio (FER) and protein efficiency ratio (PER) of juvenile Jian carp.

Our data are in line with McCormick (2010) (23) who stated that riboflavin is vital for growth, improves the metabolism of carbohydrates, fats, and protein.

Yates *et al.,* (2003) (24) also reported that riboflavin can improve the performance of the gastrointestinal tract of rat, and in turn improvement the process of digestibility and absorption. Moreover, Rivlin (2010) (25) proved that severe riboflavin deficiency can impair the metabolism of other nutrients especially B vitamins, therefore inadequate intake of riboflavin would be expected to cause disturbances in steps in intermediary metabolism, with functional implications.

Our results showed that no significant changes was observed of body weight gain, and feed efficiency ratio of rats fed tested diet enriched with microbial riboflavin as compared to negative control containing synthetic riboflavin; this is may be due to the presence of tannic acid in the tested diet. Afsana *et al*.,(2004) (26) confirmed this result by reporting that tannic acid have the ability to reduce growth, bound with protein and inhibited digestive enzymes in rats.

Effect of experimental diets on relative organs weight in adult rats suffering from iron deficiency anemia was illustrated in Table (3).

Table (3): Relative of organs weight of the different experimental groups

Parameters / Treatments	Liver (%)	Spleen (%)	Kidney (%)
G-1 Negative Control	3.86 ± 0.11a	0.53 ± 0.05a	0.89± 0.029a
G-2 Positive control	3.03± 0.11b	0.34 ± 0.01b	0.77± 0.028b
G-3 Tested group	3.25 ±0.08b	0.43 ± 0.03ab	0.80 ± 0.030a

All values represented as mean ±S.E.

Mean values at the same column sharing the same superscript letters are not significantly different (P<0.05)

As shown in this table, it could be noticed that relative weights of liver, spleen and kidney in anemic positive control group recorded significant decrease compared with negative control.

Meanwhile, relative liver weight of rats fed tested diet showed no significant change as compared to positive control and significant change compared to negative control, while relative kidney weight of rats fed tested diet showed significant change as compared to positive control and no significant change as compared to negative control.

The marked decrease in relative organs weight noticed in positive control group can be attributed to body weight loss. These findings are to a large extent in agreement with Diaz-Castro *et al.,* (2008)(27) who reported that the body weight of the anemic rats were significantly lower, whereas the liver weight was slightly decrease in anemic rats compared with negative control.

Data presented in Table (4) showed the concentrations of hemoglobin, hematocrit and read blood cells (R.B.Cs) of negative control, positive control and tested group. The mean values of hemoglobin were (15.85 ± 1.18, 10.62 ± 0.65 and 16.92 ±0.39 g/dl respectively). For hematocrit the mean values were (47.56± 3.56, 31.87± 1.95 and 50.78± 1.19 %. respectively) while, the mean values of R.B.Cs were (7.921 ± 0.59, 5.31 ± 0.32 and 8.42 ± 0.198 respectively)

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Table (4): haematocrit, hemoglobin and R.B.CS of the different experimental groups

parameters / Treatments	Hemoglobin g/dl	Hematocrit (%)	R.B.Cs ($x10^6$ul)
G-1 Negative Control	15.85 ± 1.18a	47.56± 3.56a	7.921 ± 0.59a
G-2 Positive control	10.62 ± 0.65b	31.87± 1.95b	5.31 ± 0.32b
G-3 Tested group	16.92 ±0.39a	50.78± 1.19a	8.42 ± 0.198a

All values represented as mean ±S.E.

Mean values at the same column sharing the same superscript letters are not significantly different (P<0.05)

Our results showed that hemoglobin, hematocrit and R.B.Cs of rats fed tested diet showed high significant increase as compared to positive control. No significant differences were found in all parameters as compared to negative control group.

Afsana *et al.,* (2003) (28) confirmed our results by reporting that dietary tannic acid reduced iron absorption, thereby inducing severe iron-deficiency anemia.

These finding are in harmony with those of Afsana *et al.,* (2004) (26) and Mahmoud *et al.,* (2012) (29) who stated that tannic acid has harmful effects on the two major components in the structure of blood cells, i.e protein and iron.

In the same time, Fishman *et al*., (2000) (3) reported that feeding rats with dietary riboflavin consistently increased iron absorption and therefore, partially improved the hemoglobin concentrations and hematocrit levels which reduced by TA. He also added that riboflavin deficiency may impair iron absorption, increase the rate of gastrointestinal iron loss, and/or impair iron utilization for the synthesis of hemoglobin (Hb).

According to Powers (2003)(30) and Rivlin *et al.,* (2010)(25) vitamin B2 is responsible for maintaining healthy blood cells and deficiency of riboflavin impairs iron absorption and can develop anemia.

Moreover, Ibrahim *et al.,* (2011)(31) concluded that intake of iron-folic acid tablets during pregnancy increases hemoglobin levels and in turn prevents and treatment of anemia.

Moreover Fishman *et al*., (2000)(3) reported that folic acid deficiency contributes to anemia primarily by disrupting cell division which compromises erythropoiesis. Folic acid supplementation can present magaloblastic erythropoiesis among severly folate deficient individuals, but the extent to which this translates into increases in Hb & HCT concentrations of public health importance.

Data presented in Table (5) showed the concentrations of serum iron, total iron binding capacity, transferrin saturation % and ferritin of negative control, positive control and tested groups. As shown in this table, the serum iron of normal rats (-ve control) used in the experiment amounted to 152.45 ± 7.16 µg/dl. These rats when rendered anemic had a serum iron value of 88.88 ± 7.02 µg/dl. The value of serum iron of the treated tested group was 151.04 ± 8.04 µg/dl. It could be noticed that tested formula improved serum iron level significantly as compared to the anemic group and it reached a value near to that of normal rats.

Table (5): Serum iron & Total iron binding capacity & Transferrin saturation(%) and Ferritin of the different experimental groups

parameters / Treatments	Serum iron (µg/dl)	Total iron binding capacity (µg/dl)	Transferrin saturation (%)	Ferritin (µg/l)
G-1 Negative Control	152.45±7.16a	593.82±18.45 b	25.78 ± 1.48a	193.28 ± 2.78a
G-2 Positive control	88.88 ±7.02b	684.98±14.95 a	12.85± 0.90b	149.98 ± 1.91c
G-3 Tested group	151.04±8.04a	594.77±11.56 b	25.52± 1.70a	176.41 ± 2.61b

All values represented as mean ±S.E.

Mean values at the same column sharing the same superscript letters are not significantly different (P<0.05)

The value of total iron binding capacity of anemic positive control group significantly increased compared to healthy negative group. (684.98 ±14.95 μg/dl, 593.82 ± 18.45 μg/dl respectively). As a result of feeding anemic rats with the tested formula which contain the natural riboflavin and folic acid, the value of TIBC improved significantly (from 684.98 ± 14.95 mg/dl to 594.77 ± 11.56 μg/dl respectively).

The percentage saturation ranged between 25.78% for negative control, 12.85% for anemic positive control and 25.52% for tasted groups.

Serum ferritin level significantly decreased in anemic group compared with negative control group and improved significantly in tested group (149.98 ± 1.191, 193.28 ± 2.78, 176.41 ± 2.61 μg/dl respectively).

As a result of reducing iron absorption, tannic acid-containing diet (+ve control group) as shown in the present study lowered serum iron significantly. The same effect was noticed regarding both ferritin and transferrin saturation% in serum. In contrast serum TIBC was increased significantly. These findings are in agreement with those of Mahmoud *et al.,* (2012) (29).

The positive correlation between both iron and ferritin was explained by Andrews *et al.,* (1994)(32) who mentioned that serum ferritin concentration correlates with tissue iron stores. So, serum ferritin is probably the most reliable indicator of total body iron stores in large species. The situation is different with respect to TIBC, i.e: its level is elevated when both serum iron and transferrin saturation are low (33).

Ai *et al.,* (2008) (34) and yates *et al*., (2003) (24) confirmed our results by stating that riboflavin is thought to improve iron uptake and absorption by improving the gasterointestinal function and better appetite which may have resulted in significantly enhanced concentration of serum iron.

Also Meng *et al*., (2009) (35) reported that inadequate riboflavin intake is an important risk factor for anemia. These findings are in keeping with the known biological role of riboflavin in enhancing iron absorption and utilization. When riboflavin intake is high the ability to mobilise iron from ferritin to and utilize it for the synthesis of hemoglobin will be high (36).

Moreover vitamin c deficiency has been associated with various forms of anemia. In its role as a reducing agent vitamin c can facilitate iron absorption from the gastrointestinal tract and enable its mobilization from storage (3).

Data presented in Table (6) showed the mean values of malondialdehyde (MDA) and total antioxidant (TAC) capacity of negative control rats, positive control and tested group. The mean values of malondialdehyde were (2.345 ± 0.22, 4.071 ± 0.034 and 1.92 ± 0.08nmol/ml respectively). For total antioxidant the mean values were (2.04 ± 0.22, 1.45 ± 0.15 and 2.18 ± 0.10 mM/L, respectively).

Table (6): Serum Malondialdehyde and total antioxidant capacity of the different experimental groups

parameters / Treatments	Malondialdhyde (nmol/ml)	Total antioxidant (mMl/L)
G-1 Negative Control	2.345± 0.22b	2.04 ± 0.22a
G-2 Positive control	4.071 ± 0.034a	1.45 ± 0.15b
G-3 Tested group	1.92 ± 0.08b	2.18 ± 0.10a

All values represented as mean ±S.E.

Mean values at the same column sharing the same superscript letters are not significantly different (P<0.05)

These data showed significant higher value of malondialdehyde and significant lower value of total antioxidant capacity of positive control group (anemic group) compared to negative control group. On the other hand, noticeable improvement in MDA and TAC levels were observed by feeding rats with tested formula compared with the positive control group Table (6).

The production of free radicals is the initial step in a chain of events that eventually leads to membrane lipid peroxidation.

MDA and TAC which are indices of oxidative stress were ameliorated by feeding rats with tested diet that producing folic acid. These results are in agreement with Ebaid *et al*., 2013(37) who suggested that folic acid can effectively protect against lipid peroxidation.

Besides strawberry contains fat soluble vitamins, including carotenoids, vitamin A, vitamin E, vitamin C and vitamin K and high content of vitamin C. Also it considered a sufficiently good source of other vitamins such as riboflavin, niacin, thiamin and vitamin B6 (38).

Strawberry extract were found to have high antioxidant activity. The antioxidant properties of strawberry has been shown to be mainly due to high content of phenolic compounds more than to vitamin C (39,40). Moreover, our results are in concordance to similar finding reported by Wang and Jiao (2000)(41) who confirmed that strawberry extract exhibited a high level of antioxidant capacity against free radical species.

Our results were obviously confirmed by histopathological examination (Figures 1-3).

Normal spleen tissue showed white pulp surrounded by red pulp separated by splenic tissue fibrous trabecular. By contrast spleen tissue of the anemic rats showed disturbed splenic pulps, with congested blood vessels and lymphocytic infiltration around the central vein. Our results are in agreement with those of Hamada et al., (1998) (42) These changes were markedly restored by the tested formula.

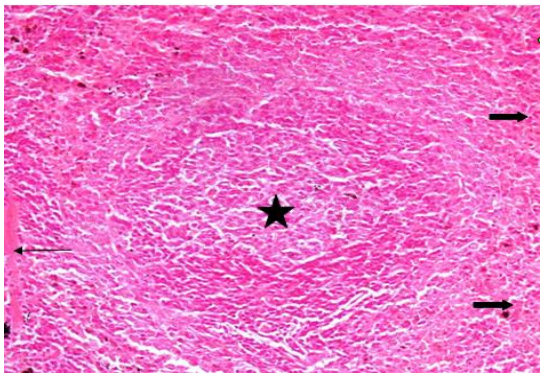

FIG1 : A photomicrography of splenic tissue for the control group which consist of white pulp (black star) surrounded by red pulp (thick black arrow) and separated by splenic fibrous trabecula(thin black arrow) (Hx.&E. X200).

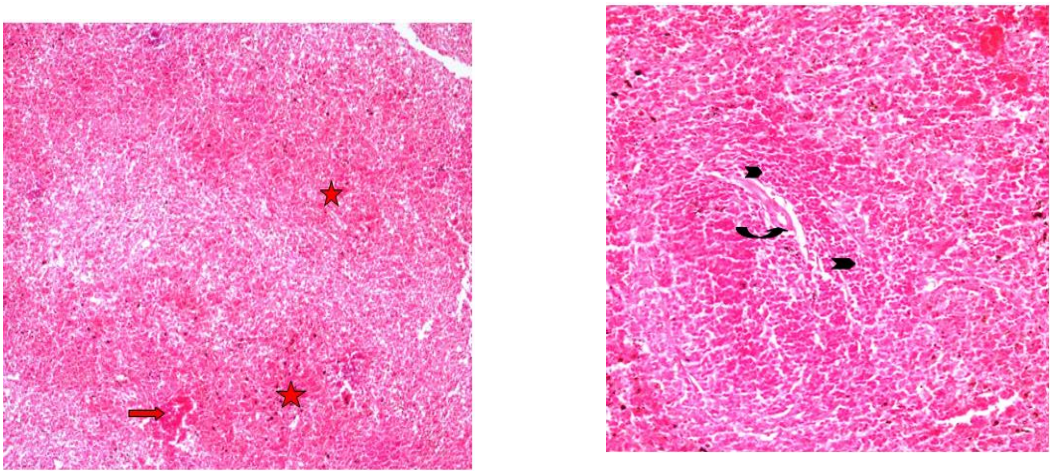

FIG2: A photomicrography of splenic tissue for the second group (diseased)showing in the left one disturbed splenic pulps(red stars) with congested blood vessels(red arrow), And on the right one with higher magnification showing lymphocytic infiltration(black arrow heads) around the central vein(curved black arrow). (Hx.&E X100&200).

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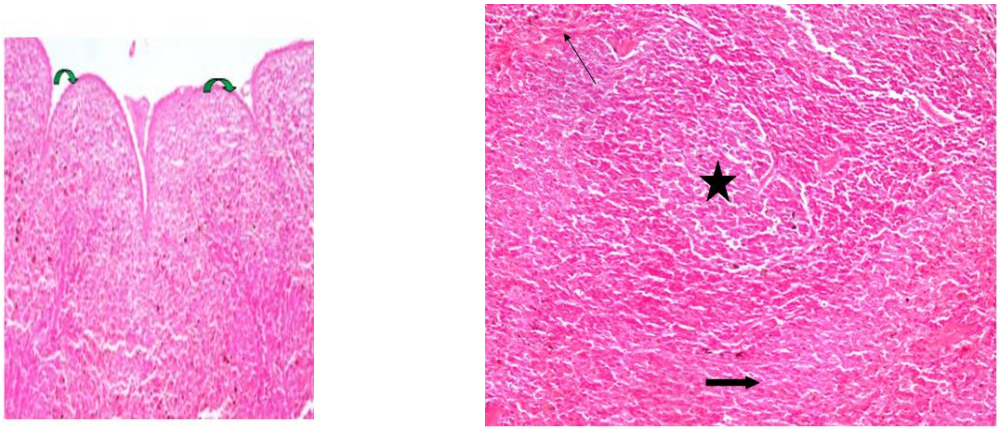

FIG3:A photomicrography of splenic tissue for the third group (treated)showing in the left one well formed and notched splenic capsule (green curved arrow) and in the right one with higher magnification showing well formed white splenic pulp (black star)and red splenic pulp (black thick arrow) and separated by the splenic trabeculam (thin black arrow). (Hx.&E X 100&200).

CONCLUSION

It can be concluded from the present study that tested formula containing probiotic that produce folic acid and riboflavin afforded modulatory effects against tannic acid induced anemia as manifested by amelioration of blood hemoglobin, serum iron, total iron binding capacity, ferritin, lipid peroxidation and total antioxidant capacity. Our biochemical results were supported by histopathological examination. We recommend to use probiotics as food components in the protection of various disorders of anemia.

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