THE INFLUENCE OF THE BREAD MAKING PROCEDURE ON THE IRON BIOAVAILABILITY

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Abstract: The aim of the present study was the investigation of the influence of the bread making method on the iron bioavailability in the iron fortified bread. The enzymatic degradation of the phytates ($InsP_6$) was studied within gastro-intestinal digestion conditions *in vitro.* The study of the iron bioavailability was drowning *in vitro*, according to the Monsen model, and *in vivo* on white laboratory rats. The study of the biochemical indices of the blood, collected from the laboratory animals, fed with fortified bread with 8 mg Fe/100 g product, made by the traditional method and by the lactic-acid fermentation method compared to the control group showed that iron intake plays conclusive role in animal nutrition. Thus, iron statute of both experimental groups, and especially iron reticence in the body, was essentially improved compared to the control group.

Keywords: *iron deficiency anemia, food fortification, bread, phytic acid, bread making, acid-lactic fermentation, in vitro, in vivo study, peripheral blood parameters, iron statute*

INTRODUCTION

According to WHO criteria, anemia in the Republic of Moldova represents a major public health problem [1]. The results of the investigations drown under UNICEF supervision over a group of 792 children under 5 years old and their mothers show anemia presence at 47% of children between 6 and 12 months old, at 28% of children under 5 years old and at 40% of women of fertile age. According to the statistics of the Ministry of Health, anemia rate in the case of children till one year old constitutes 20%. It is common knowledge that one anemia case corresponds to one case of iron nutritional deficiency, it is possible to assume that approximately half of the children under 5 years old and 40% of fertile age women have iron deficiency. Prevalence of blood and hematopoetic system diseases increased during last 5 years with 46.4% and for anemia with 50.3% [2].

Iron, although present in the body in small amounts (0.005% of body weight) plays key roles in many biological functions. Iron is presented in human body in two forms: hemic iron, incorporated in heme structure (approximately 65%, thus 2-2.5 g of total iron present in the body) which is part of the hemoglobin, myoglobin and protein enzymes, and non-heme iron, present in the composition of some non heme enzymes, transfer in as well as reserve iron of the body which may vary from 300 to 1200 mg. Physiological needs in iron vary depending on the age: from 0.72 mg/day for children under one year old and 2 mg/day for mature women. But these requirements must be adjusted according to the iron absorption which varies essentially depending on the food composition.

The study of food consumption and nutritional intake of families in the Republic of Moldova showed that the products presented in the daily ration of 18-45 years old women contain just 53% from the required iron amount (adjusted to the absorption rate), and in case of the poor categories of population it reaches only 23% of the daily needs. A recent study of the food intake [3] which aimed the estimation of the nutritional statute of the institutionalized children of 11-17 years old in Republic of Moldova marked out the fact that the dialyzable iron intake is extremely low and constitutes just 0.87 mg Fe/1000 kcal (reference index is 4.67mg Fe/1000 kcal). Medium iron intake does not reach 100% level of the nutritional requirements in any population category [3].

Malnutrition and deficiency of mineral origin micronutrients is not the only responsible factor for these problems. In the Republic of Moldova the alimentation consists of mainly vegetal products, from which iron is insufficiently assimilated. This is the reason why even in cases when the nutritional support is sufficient and complete, for the majority of population chronic iron and calcium deficiency persists. This leads to predisposal of human body to different nutritional diseases. One of the causes of calcium, iron, and zinc deficiency is the impact of some antinutrients – phytates, tannins and other fibers, which essentially reduce biological assimilation of these micronutrients through their complexation [4]. Thus, administration of these deficient elements as food supplements is often insufficient.

One of largely used methods in the highly developed countries is food fortification with deficient micronutrients [5]. This method is easily realized and has very low cost (in the case of the mineral micronutrients) [6]. Refining of cereal products leads to an

important loss of minerals and fibers and may have important consequences on such disease as colon cancer, diabetes mellitus and heart diseases. Flour fortification with micronutrients represents an outspread procedure which is aiming the reinstatement of micronutrient composition affected during processing. Thus, if non milled wheat contains approximately 33 $mg.kg^{-1}$ iron, after milling this amount is reduced to 11 $mg.kg^{-1}$. Administration of 20 $mg.kg^{-1}$ iron to the flour will confer its natural qualities. However, food fortification cannot be considered as a simple mechanical administration of the supplements [7].

In the case of vegetal products or combined nutrition (animal origin products consumed together with the cereals) the main cause for the demineralization is considered to be phytic acid (myo-inositol hexafosfat $InsP_6$) naturally found in wheat grain as soluble sodium and potassium salts [8]. During food processing of cereal products or during digestive stage $InsP_6$ is capable to fix metal cations and can form stable structures which are not able to diffuse through gastrointestinal wall [9]. One mmol of $InsP_6$ is able to fix up to 6 mmol of bivalent metallic cations (Fe, Ca, Zn). This contributes to an essential demineralization of food in the case of high intake of fibers and cereals. During food fortification, processing and storage, numerous physico-chemical and enzymatic processes take place which may greatly influence biological value of these products. Hereby, elaboration of a technologic process of fabrication of fortified products should be based on a profound study of the evolution of these micronutrients during technological process as well as during consumption.

The aim of the present study is investigation of the influence of bread making method on iron bioavailability from bread fortified with iron. The process of enzymatic degradation of phytates ($InsP_6$) during gastro-intestinal digestion and *in vitro* was studied as well. The study of iron bioavailability was performed *in vitro*, according to the Monsen model and *in vivo* on white laboratory rats.

MATERIALS AND METHODS

During the study, we used bread made from superior quality wheat flour of autochthon production (standard STAS-26574/85). As iron additive, iron(II) sulfate ($FeSO_4 \cdot 7H_2O$) was used, which is accepted as food additive by "Codex Alimentarius" in the Republic of Moldova.

Bread making procedures

Bread was made by direct (mono-phase) method, which comprises the concurrent mixing of all the ingredients; indirect (bi-phase) method with the administration of additive at the kneading stage and by lactic-acid method.

To reproduce the fabrication of integral wheat bread, the integral reconstituted wheat flour was used (flour plus bran). For the preparation of bread with yeast, 6 kg of integral wheat flour (4.65 kg flour plus 1.35 kg bran) were mixed with 3.6 L distilled water and 150 g of yeast (*Saccharomyces cerevisiae*). For lactic-acid fermentation, the dough was prepared by the mixing of 1.2 kg integral wheat flour (0.93 kg flour plus 0.27 kg bran)

with 600 mL distilled water. After one day of natural fermentation at 30 °C, the dough was mixed with 4.8 kg of integral flour and 3 L of distilled water.
For bread prepared by lactic-acid method with yeast addition, the ingredients were mixed during 5 minutes. The obtained dough was stored at 30 °C to allow fermentation to start. Then the dough was knitted again for the gluten formation and left at 30 °C. The dough was backed after 3 hours of fermentation for the bread with yeast and one hour for dough made by lactic-acid method. Bread was lyophilized, milled and introduced in the alimentation of rats.

In vitro analysis procedures
Iron *in vitro* digestibility, as well as investigation of enzymatic degradation process of phytates was performed in two stages: gastric stage (*pH* = 2, in pepsin presence) and intestinal stage (*pH* = 8.2, in trypsin presence) [11, 12]. 10 g sample were stored at 37± 0.1 °C for 15 min in acidic environment (*pH* = 2) created by addition of 1.5N HCl solution. After pepsin administration (150 mg/100 g product) the blend was incubated for two hours at 37±0.1 °C with continuous mixing. Each 30 min equal samples were taken for the analysis, centrifuged for 10 min (6000 $rot.min^{-1}$). The intestinal stage was realized in the same conditions after the establishment of the *pH* value at 8.2, in trypsin presence in $NaHCO_3$ 0.08 M environment. The amount of soluble iron was determined through the spectrophotometric method [13].
Phytic acid amount ($InsP_6$) in the flour and the product was determined through the spectrophotometric method [9]. The method is based on the organic phosphates capacity to interact with the ammonium molybdate and vanadate with formation of a yellowish – gold complex. The intensity of the color is correlated with phosphorus amount. Subsequently the amount of $InsP_6$ was recalculated since phytic phosphor constitutes just 28.2% of the total $InsP_6$.
The analysis were made using DR-5000 spectrophotometer, all tests were made in triplicates.

In vivo analysis procedures
To analyze the influence of the diet with different iron amounts (bread prepared by different procedures with iron addition) on the main hematological indexes the study on a sample of 20 white Wistar rats with the body weight of 180-210 g divided in 3 groups (6-7 in each group) was designed. The animals from the first group (control sample) were fed with ordinary non fortified bread (bread prepared by classical and bi-phase method). The second group comprised the rats fed with bread prepared through traditional, bi-phase method enriched with iron (bread prepared through the traditional, bi-phase method with 80 mg/kg iron addition). The third group comprised the rats fed with bread prepared by lactic-acid fermentation method enriched with iron (80 mg Fe/kg). Animals from all three groups were kept on the above mentioned diet for 21 days.

Models of samples obtaining and analytic procedures
Biological material – peripheral blood from the laboratory animals was obtained twice – initially, before the beginning of the experiment and at the end of the experiment through the procedure described by Ciudin *et al.* [19]. Blood was collected to single use

test tubes Eppendorf type of 1.5 mL. Blood indexes have been appreciated in the heparinizated peripheral blood in the hematologic analyzer PCE-210 (ERMA, Japan).
Blood serum was obtained through centrifugation of the peripheral blood at 3000-4000 rot/min for 15 minutes. After centrifugation, blood serum was transferred in Eppendorf test tubes and was stored in the refrigerator at +4 ºC till the end of the experiment. Blood iron level determination was performed using the method with cromasorol according to the working instructions of the analyzer ("Iron Cromazurol", "Eliteh", France).

EXPERIMENTAL RESULTS

Production processes of cereal products (flour moistening, fermentation and dough knitting, backing and drying) influence greatly on the amount of phytates in food products. Hereby, vegetal phytase, an enzyme that degrades phytic acid and its soluble sodium and potassium salts, reduces essentially the amount of this antinutriment during technological processing of the flour especially during fermentation. This process is explained by the establishment of optimal conditions for development of phytase activity during dough fermentation – at 35-40 ºC, *pH* value 4-5, as well as due to the fact that bread yeast amplify this process through their own phytase activity [7].
However, recent use of fast bread making procedures, as well as the use of the iron supplements at the beginning of the dough fermentation leads to rapid fixation of bivalent metal cations in chelate compounds such as $InsP_6 \times Fe$, subsequently not capable to diffuse the gastro-intestinal wall and they are not subjected to enzymolysis.
In order to demonstrate the influence of bread making procedure (type and duration of fermentation, bread yeast quality), the results obtained during application of traditional bread making method – mono-phase and bi-phase method as well as of lactic-acid fermentation method based on the use of the wheat bran reconstituted through the addition of bran were compared (table 1).

In vitro procedures

It was established that during mono-phase method, only 15% phytates presented in the bread were subjected to hydrolysis during 2 hours of *in vitro* gastro-intestinal digestion. Soluble iron amount (dialyzable) was, at the end of these 2 hours of gastro-intestinal digestion, 0.02 mg/100 g product, which constitutes just 4.6% of the total iron naturally present in the product (2.5 mg/100 g product).
Phytates in the bread made by bi-phase method underwent hydrolysis in 36.5%. In this case, the amount of the dialyzable iron constituted 0.2 mg/100 g product, or approximately 8% from the total iron amount. Obviously, this difference is due to bread making procedure, because the ingredients composition is the same in all the cases. In the first case, fermentation procedure goes rapidly while during bi-phase procedure in the starter an important phytase activity will develop due to the phytase present in the flour as well as due to the enzymes present in the bread yeast.

Table 1. *The influence of bread making procedure and the recipe on the iron bioavailability* in bread products***

Bread making method	Additive, mg Fe/ 100g product	Total ferrous, mg Fe/ 100 g product	Bread yeast %	$Fe_{soluble}$		**Phytates $_{soluble}$	
				mg/100 g product	%	mg/100 g product	%
Monophasic	-	3.26 ± 0.23	100	0.14 ± 0.02	4.6	110.6 ± 13	14.60
Biphasic	-	3.26 ± 0.23	100	0.17 ± 0.03	5.1	155.5 ± 29	20.52
Monophasic	4	7.02 ± 0.37	100	0.78 ± 0.15	11.2	92.7 ± 19	12.23
	8	10.12 ± 0.39	100	1.17 ± 0.11	10.8	91.6 ± 11	12.09
Biphasic	4	6.82 ± 0.25	100	0.94 ± 0.13	13.8	147.7 ± 31	19.49
	8	10.57 ± 0.23	100	1.35 ± 0.16	12.8	130.8 ± 28	17.26
Biphasic with lactic-acid fermentation	4	6.96 ± 0.37	0	2.00 ± 0.13	28.7	372.1 ± 41	49.08
	8	10.29 ± 0.31	0	2.77 ± 0.26	26.9	365.4 ± 23	48.21
	4	7.01 ± 0.21	25	2.39 ± 0.36	34.2	398.0 ± 31	52.5
	8	10.52 ± 0.23	25	3.23 ± 0.46	30.7	394.5 ± 17	51.4
	4	7.09 ± 0.17	50	2.52 ± 0.43	35.5	406.6 ± 41	53.9
	8	10.68 ± 0.13	50	3.43 ± 0.27	32.1	413.9 ± 56	54.6
	4	6.67 ± 0.16	75	2.31 ± 0.19	34.7	383.6 ± 34	50.6
	8	9.52 ± 0.33	75	2.97 ± 0.16	31.2	394.9 ± 29	52.1

* - Fe total – 2.5 ± 0.2 mg/100 g product

** phytates –total phytates amount in the product 578.0 ± 5.0 mg phytic acid/100 g product

The administration of the flour at the dough formation stage is followed by a considerable phytate enzymatic hydrolysis, which leads to a more intense release of the iron from the insoluble phytate complexes.

A number of previous studies showed that lactic-acid fermentation of the dough, established using bran from the reconstituted wheat flour, contributes greatly to an essential decrease of the phytate amount [8]. Lactic-acid micro flora, isolated from the dough obtained through natural fermentation is capable to degrade phytates. Together, phytic degradation and formation of lactic acid could contribute to a more intense release of the minerals in the products.

Hereby, during this study the influence of lactic-acid fermentation, established on the fasts of bran from the reconstituted wheat flour, was investigated. In order to show the important role of yeast on this process, bread products were made. After lactic-acid fermentation development (with the duration of fermentation of 24 hours) into the recipe (prescription) for traditional (bi-phase) method respectively 25, 50 and 75% of yeast was added.

According to obtained results, in the case of application of the natural dough fermentation procedure, 50-57% of the present phytates were hydrolyzed after 2 hours *in vitro* gastro-intestinal digestion. The addition of the bread yeast does not seem to have an important effect (table 1). This shows that even though phytate decrease during traditional bread making procedure was identified, this cannot be referred to the phytase activity of the bread yeast but is due to endogen flour phytase activity.

Dialyzable iron amount in this case is higher (0.34 – 0.56 mg/100 g product), which constitutes 13-22% from the total iron present in the product. This is obviously due to an important degradation of phytates, which makes this mineral insoluble. Apparently,

the naturally present iron intake from wheat flour is insufficient for diets, which comprise few products that contain hemic iron.
The study of iron bioavailability in the fortified bread products, prepared through the bi-phase and lactic-acid method shows that in the case of bi-phase method only 8-12% of the present iron is presented in its soluble form after 2 hours of gastro-intestinal digestion (table 1). In the case of bread prepared by lactic-acid method, the rate of the dialyzable iron constitutes 20-22%.
It is obvious that *in vitro* investigations do not reflect the multitude of the factors that may influence the mineral valorization in the gastro-intestinal tract. First, there are subjective factors that reflect body reserve of iron. According to the literature data, *in vitro* bioavailability is related with *in vivo* digestion in a proportion of 60-76% [8, 12].

In vivo procedures

The analysis of the peripheral blood parameters collected from the animals

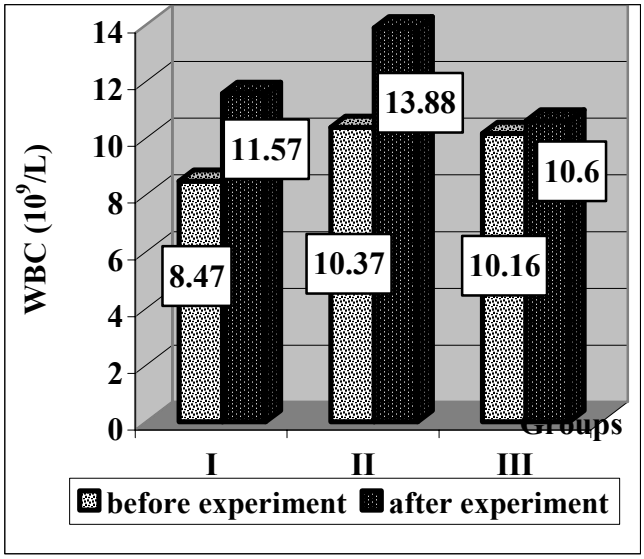

Figure 1. *Modification of leukocyte number for the animals without anemia before and after investigations*

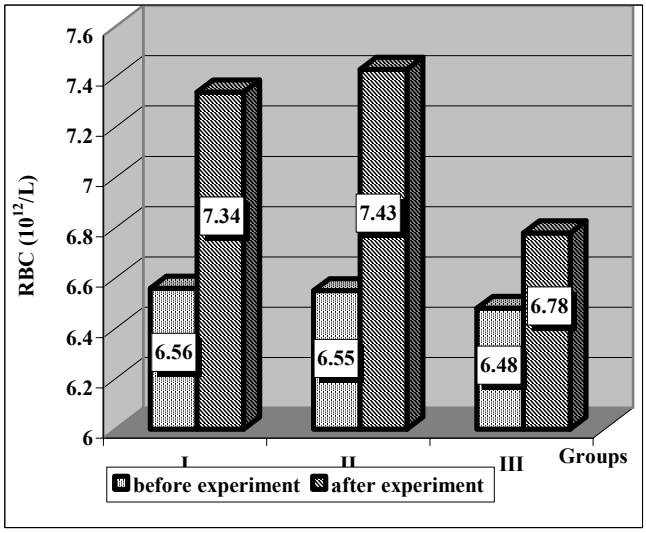

Figure 2. *Modification of erythrocyte number (RBC parameter) before and after investigations*

The leukocyte number in 10^9/L (WBC) increases in case of animals from control group (group I) and those fed with bread fortified with iron prepared by bi-phase method (group II). In case of group fed with bread prepared by lactic-acid method (group III), leukocyte number slightly diversifies (figure 1). Erythrocyte number (RBC) in 10^{12}/L increases for the control group (I) as well as for the groups fed with fortified bread (II and III) but this variability is more essential for the animals fed with fortified bread prepared by lactic-acid method (figure 2).

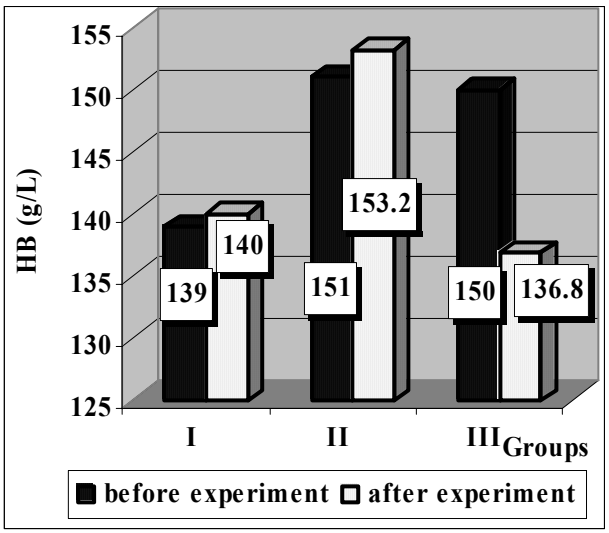

Figure 3. *Modifications of the hemoglobin amount (HB parameter) before and after experiment*

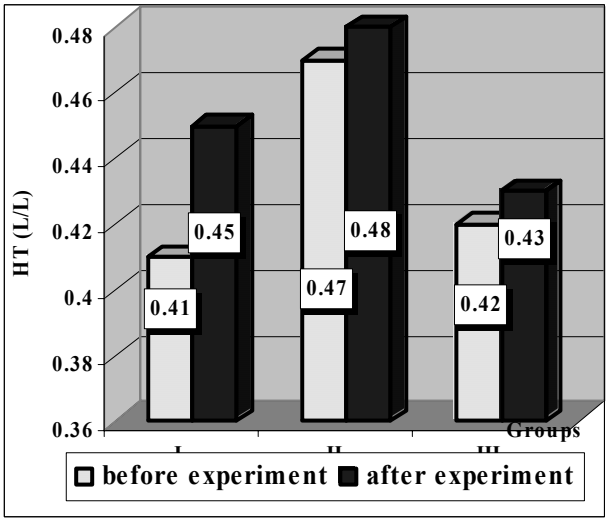

Figure 4. *Modifications of the hematocrit (HCT parameter) before and after investigations*

Hemoglobin (Hb) insignificantly varies in the examined three groups of rats (figure 3). However, for all three groups a tendency for HB increase was noticed, which is more significant for the rat groups fed with fortified products (an increase of 2.2 and 2.9 $g.L^{-1}$ respectively for the groups fed with fortified bread prepared by classic method and lactic-acid fermentation method).

Hematocrit (Ht) corresponds to the volume occupied by the red globules reported to the total blood volume (L/L), providing, thus, information about the circulating hemoglobin concentration. For control group as well as for the animal groups fed with iron fortified

bread, Ht increases insignificantly and reaches values of 0.44-0.48, which corresponds to the established standard values (figure 4).

Average volume of an erythrocyte (MCV) is an extremely important globular characteristic, which gives information about the severity and etiology of anemia. Microcitosis is associated with an insufficient hemoglobin amount and foregoes, normally, a significant decrease of the circulant hemoglobin level. In this case, the average volume of the erythrocytes increased with about 22-26 fl for the control group as well as for the animal groups fed with fortified bread. Investigated parameter indicates a satisfactory state of the blood for all three examined groups.

Average hemoglobin amount in an erythrocyte (MCH) insignificantly decreases for the control group (with 2 pg), while for the animal groups fed with fortified bread it increases with 2.2-3.2 pg. Average corpuscular concentration of hemoglobin (MCHC) represents a morphologic characteristic, extremely important for the red globules. Platelets (decrease of hemoglobin concentration), it is an indicator of the severe anemia. Present investigations testify an essential increase of average corpuscular hemoglobin concentration for the control group as well as for the groups fed with fortified bread. Obviously, for the II and III group the increase is more evident, which is due to a more important iron intake.

Platelets number in 10^9/L (PLT) increases for all three investigated groups, but for the II and III group the increase is significantly higher. Hereby, if for the control group an increase with 98 unites was observed, for the II group PLT increased with 181 unites, and for the III group with 251 unites. This increase indicates an essential improve of iron statute for the animal groups fed with fortified bread. The percent proportion of the lymphocytes and total leukocytes (LYM, %) increases insignificantly for all three examined groups. Absolute lymphocyte number (LYM, 10^9/L) increases essentially for the control group – with 3.1×10^9/L, and for the II and III groups there are significant changes. The Price-Jones curve, which indicates the amplitude of erythrocyte distribution (RDW, fl) indicates a significant increase of the above-mentioned parameter, especially for II and III group. The distribution amplitude of the platelets (PDW, fl) does not vary for the control groups, while for the experimental groups II and III a decrease of this particular parameter was established.

An average platelets volume (MPV, fl) increases for the control group with 1.3 fl, for the II group with 0.5 fl and for the III group decreases with 0.8 fl. The ratio of the big platelets, with 12 fl diameter towards the total platelets volume (P-LCT) significantly decreases, especially for the III group, where this particular parameter decreases almost twice.

Analysis of blood iron transport indicators

Serum iron concentration is an indicator of the amount of iron in plasma, fixed to a specific protein – transferin. The majority of iron which is part of plasma derives through catabolism of reticulo-endotelial system, and iron that leaves plasma derives from bone marrow. Regardless the fact that plasmatic iron reserve is not significant (approximately 3 mg), the last is very active because it transports daily about 30 mg of iron. Transport parameters of the generally are not modified till body reserve of iron is not completely depleted. In the convalescence period, after a severe anemia, namely serum iron is indicator of recovery degree and of the body iron statute.

It was established, in the experimental groups, that after the administration of the special diet the level of serum iron was of 36.48±13.8 μmol.L^{-1} for the control group (I), of 41.55±19.58 μmol.L^{-1} (II group) and of 40.14±18.78 μmol.L^{-1} for group III (fig. 5).

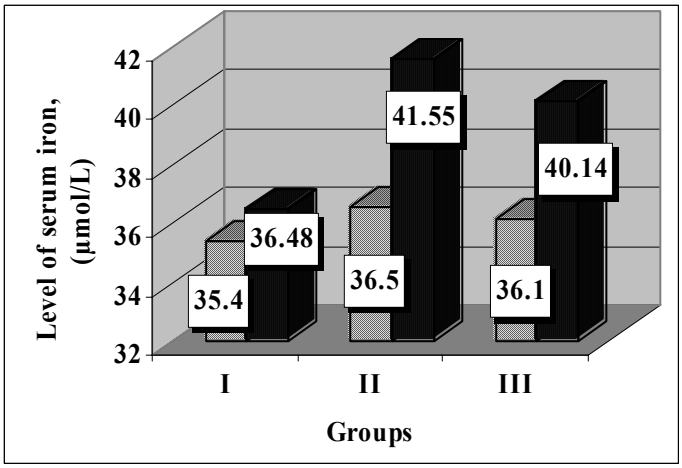

Figure 5. *Serum iron evolution after the application of the special food:*
I-control group;
II- fortified bread produced through traditional method;
III- fortified bread produced by lactic-acid method

Analysis of leukocyte formula of the blood collected from the animals without and with anemia

Leukocyte formula of blood collected from the rats from the I-III groups before and after administration of the special diet is given in table 2.

Table 2. *Leukocyte formula for the animals before and after special diet administration*

Blood indices	Studied animal groups		
	I	II	III
Before special food administration			
Leucocytes, G/L	8.47±0.68	10.37±0.91	10.16±1.01
Young cells (mielocites, metamielocites, %)	0	0	0
Neutrophyle unsegmented, %	0.7±0.50	0.5±0.30	0.7±0.33
Neutrophyle segmented, %	33.0±2.0	28.0±2.0	28.0±2.0
Eozinophyle, %	0.9±0.5	0.7±0.3	1.2±0.4
Basophyile, %	0.7±0.3	0.5±0.3	0.7±0.3
Lymphocyte, %	56.7±3.1	63.0±3.0	61.6±2.0
Monocyte, %	7.0±0.61	7.3±0.4 1	7.8±0.72
Normocyte	-	-	-
After special food administration			
Leucocytes, G/L	11.57±0.68	13.88±0.91	10.60±1.01
Young cells (mielocites, metamielocites, %)	0.83±0.9	0.68±0.9	1.67±0.99
Neutrophyle unsegmented, %	0.8±0.50	0.8±0.40	0.8±0.4
Neutrophyle segmented, %	30.0±1.7	31.0±3.0	30.0±2.1
Eozinophyle, %	0.5±0.1	1.7±0.3	0.7±0.3
Basophyile, %	1.5±0.1	1.0±0.4	0.8±0.4
Lymphocyte, %	56.1±3.1	56.0±3.0	57.0±2.0
Monocyte, %	10.3±0.8	8.8±1.1	9.1±0.90
Normocyte	-	-	-

The analysis of the obtained results shows, that in the leukocyte formula for the intact animals after the special diet administration some specific modifications take place, and namely: a slight increase of the percent amount of the monocites and the formation of young forms of granulocytes – mileocytes and metamilocytes. These modifications were more evident for the animal group fed with bread prepared by lactic acid method.

DISCUSSIONS

The investigations reveal that iron intake and procedures applied for bread making have a significant importance for bioavailability of this essential mineral. Food products rich in fibers such as products made of integral wheat flour contain an important amount of phytates, which may reduce essentially iron absorption and utilization ability in the human body. Antinutritive effect of the phytic acid from cereals is manifested through its ability to chelate metal ions (Fe^{3+}, Zn^{2+}, Mg^{2+}, Ca^{2+}), at gastro-intestinal *pH* value thus forming insoluble complexes metal-phytate that makes the metal not available for absorption in the gastro-intestinal tract [21, 22]. Phytic acid also interacts with some enzymes (tripsin, pepsin, α–amylase, β-galactosidase), proteins and vitamins reducing their activity. It was established that phytates suppress activity of α–amylase through the chelating of calcium necessary for the enzymatic activity. With proteins, phytic acid forms complexes protein-phytates or protein-mineral-phytates which leads to a decrease of the solubility, digestibility and functionality of the proteins [23]. Dephytinisation (phytate extraction), performed through different procedures, increases significantly iron absorption [23, 24].

The procedures applied for the food transformation (knitting, moistening, fermentation, and backing) have an important influence on the amount of phytic acid in the final product, which may be the subject of enzymatic hydrolysis due to endogen phytase presence as well as due to exogen phytase from yeast. An essential role plays duration and food transformation conditions [25-27]. Phytase activity is optimal at *pH* value 5-5.5 and t = 55 °C, the enzyme being still active at 70 °C [28]. In the dough, together with endogen phytase of the dough, the phytase from yeast may also influence if it is added [29]. Phytic acid and phytate hydrolysis takes place at all stages of the bread making process, being influenced by: flour extraction degree, the amount of yeast used; temperature and duration of fermentation; dough *pH* value; water amount in the dough, phytate solubility especially of its salts; additive presence such as ascorbic acid and sodium bicarbonate [29]. More than that, phytase has a significant role for the phytate hydrolysis in the stomach and intestine, thus possible decrease of mineral absorption due to phytic acid resistance for digestion in are human intestine should be taken into consideration.

Numerous studies have shown that lactic-acid bacteria are able to degrade phytic acid, and lactic-acid fermentation increases Ca, Mg, Fe solubility as estimated during *in vitro* investigations [8, 20]. All together, phytic acid degradation and the lactic acid formation have improved mineral bioavailability in the bread prepared lactic-acid method.

In this study, it was established that non heme iron from the bread prepared by lactic-acid fermentation method has an increased *in vitro* bioavailability compared to the bread prepared by traditional methods (mon- and bi-phase). In the same time, a decrease of the

amount of phytic acid during *in vitro* gastro-intestinal digestion was noticed. These observations show that there is a direct relationship between potential iron bioavailability from the bread products and phytase degradation degree.
During *in vivo* investigations it was established that for the rats from all 3 groups the parameters of peripheral blood after 21 days of special diet administration were within established limits. For the II and III group, where the special diet was used (fortified bread prepared by traditional method and fortified bread prepared by lactic-acid method) an essential increase of hemoglobin, erythrocyte number, platelets number was observed compared to the control group. This is obviously due to an increased iron intake from the administered special diet. But the most significant influence was noticed in the case of the supplement administration on the serum iron level, which reflects body iron reserve. For those 2 experimental groups, fed with fortified bread, body iron reserve is considerably higher compared to the control group.
The comparison of biochemical indices of the blood for those two rat groups fed with fortified bread did not allow us to reveal the influence of bread making method. Hereby, although *in vitro* investigations showed that iron dialysability from the bread prepared by lactic-acid method is considerably higher (≈ 20%) compared to the iron dialysability from traditionally prepared bread (≈ 8%), *in vivo* investigations did not testify any significant difference for the biochemical indices for those 2 experimental groups.
The reason might probably be due to the considerable iron intake, which allowed to satisfy the requirements and to complete the body iron reserve. An essential role plays by the initial iron statute of those 3 rat groups that was within the normal limits at the beginning of the experiment.

CONCLUSIONS

Our investigations established that procedure applied for fortified bread production has a key influence on iron bioavailability degree in "*in vitro"* gastro-intestinal conditions. This fact is directly related with an advanced level of the phytate degradation from the bread, due to applied bread making procedure and also due to a longer bread-making procedure.
The study of biochemical blood indices, in samples collected from the laboratory animals fed with fortified bread (8 mg/100 g product), that was prepared by traditional and by lactic-acid methods compared to the control group, showed that the key role in animal nutrition plays iron intake. Hereby, iron statute of both experimental groups especially body iron reserve was significantly improved compared to the control group.
In case of sufficient iron consumption bread making procedure did not have any influence on iron statute in examined groups. In order to show the influence of bread-making procedure on iron status, it is necessary to investigate the influence of special diet administration on the laboratory animals with induced anemia.

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