IRON ABSORPTION IMPROVEMENT: AN ADDITIONAL HEALTH BENEFIT FOR CERTAIN PROBIOTICS, IN VITRO AND IN VIVO STUDY

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ABSTRACT

Probiotics has gained increasing interest from the scientific community due to their promising effects on health. However, there is a limited information about their potential on improving iron bioavailability. We aimed to investigate the effect of phytase producing probiotic bacteria on improving iron bioavailability, in vitro and in vivo. In the first phase a Caco-2 model was set up and ferritin formation in the monolayer was measured after addition of a phytase producing probiotic bacteria, with or without phytic acid, to the cell line.

In the second phase, an in vivo assay was conducted to evaluate the effect of probiotic on the serum iron in rats.

Results of the in vitro and in vivo assays revealed that the probiotic bacteria, significantly improved iron absorption from the mixture of phytate and iron, when compared with the control experiments.

The results of this study suggest that probiotic bacteria may improve the iron bioavailability of the foods with high content of phytic acid, probably due to the phytase activity in bacteria. Also, this model is suitable to study the iron bioavailability in the presence of probiotics.

Keywords: Iron bioavailability, Probiotics, Caco-2, Ferritin, In vivo, In vitro.

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Introduction

Iron deficiency affects about 3.5 billion people around the globe and after calorie insufficiency (hunger), is the most common nutritional problem worldwide. The world health organization (WHO) estimates that 66.4% of the pregnant women around the world suffer from anaemia(1). The amount of iron that a person absorbs depends on the total amount of heme and non-heme iron in the meal, its bioavailability, and physiological needs(2). According to Schumann et al., bioavailability is defined as “the sum of impacts that can increase or decrease the metabolic utilization of a nutrient”(3).

Unfortunately, numerous valuable nutritional sources of iron encompass limited iron bioavailability. Different foods may phytates (myo-inositol hexakis (dihydrogenphosphate), that strongly bind to iron ions and hinder iron absorption(4).

Phytases are valuable enzymes in upgrading the nutritional quality of phytate-rich foods and feeds(4). Probiotics are group of lactic acid bacteria (LAB) that beneficially effect on the human’s health(5, 6). Several reports have indicated that iron absorption improved by fermentation of food. They suggested that production of short chain fatty acids, decrease of pH, and some times, exertion of phytate degregading enzyme, may be the underlying process.
mechanism. Previous studies proved the ability of probiotics on production of phytase and organic acids. However, we did not find any study about the direct effect of probiotic bacteria, on iron absorption. The objective of this study was to evaluate whether phytase producing probiotic bacteria affect Fe uptake using Caco-2 cells as an intestinal epithelial model.

In the in vivo assay, the effect of probiotic on serum ferritin concentration of rats was evaluated.

**Materials and methods**

**Cell culture**

Caco-2 cells were purchased from ECACC (CB No: 02D052, UK) at passage number 7. Stock culture of Caco2 were maintained in the appropriate medium (see below). The media was changed every other day, and the Caco 2 cells were subcultured at approximately 80% confluency. At passage 12-22, the cells were seeded in 12-well plates at a density of 5×10⁴ cells/cm².

Standard media before the assay was Minimum Essential Medium (Sigma M 2279) with 1% non-essential amino acid solution plus 1% L-glutamine. Each medium was supplemented with 10% foetal calf serum (Lablech 4-101-500) and 1% penicillin & streptomycin (10,000 U ml⁻¹ & 10,000 mg ml⁻¹) (GIBCO 15140-122). They were fed with an appropriate medium and incubated at 37°C in humidified air (95%) and CO₂ (5%).

During the assay Iron Free Media (IFM) were prepared, containing: Minimum Essential Medium (GIBCO 41500-018) with PIPES (Sigma P1851), Penicillin 10,000 U ml⁻¹ & streptomycin 10,000 μg/ml (GIBCO 15140-122) Hydrocortisone (Sigma H0888-1G), Insulin (Sigma I1882), Sodium selenite (Sigma S5261-10G), Triiodo-L-thyronine, sodium salt (Sigma T6397-100G) and epidermal growth factor (Sigma E4127)⁴.

**Phytase activity assay**

As probiotic bacteria may excrete phytase enzyme, that its activity is strain specific, and have a strong effect on iron bioavailability, the phytase activity of L.a was measured in the bacteria free supernatant (bfs).

For preparation of the bfs, the probiotic bacteria was subcultured in 20 ml of the modified MRS broth, where the only source of phosphate was sodium phytate: Peptone (Oxoid L37) 10 g, Meat extracts (Oxoid L29) 10 g, Yeast extracts (Oxoid L21) 5 g, sodium phytate (Sigma P8810) 2 mg, Di-ammonium citrate (BDH 27153) 2 g, Glucose (Fisher Scientific, UK, G/0500/53) 20 g, Tween 80 (Prolabo L171) 1 g, Na acetate (Sigma S8750) 5 g, MgSO₄.7H₂O (BDH 29117) 0.58 g, MnSO₄.4H₂O (BDH 29146) 0.28 g, made up to 1 liter with distilled water, pH 6.2-6.4 / anaerobic/ 37°C. The medium with bacteria was centrifuged at 8000 g for 15 minutes, and supernatant was sterilized using a 0.2 µm diameter filter.

The phytase activity of the probiotic strain was tested using the “phytic acid Color Kit-complete phytase assay system” (Innova Biosciences), according to the manufacturer’s instructions.

**In vitro assay**

**The effect of L.a on iron bioavailability by Caco-2 cells**

Bioavailability of non-heme iron was determined using ferritin concentration as an index of bioavailable iron in response to iron uptake. Twelve days post-seeding, the trans-epithelial electrical resistance was measured to verify the intactness of the Caco 2 cell layers. The resistance across the layers was typically 450±60 Ω. The cells were washed three times with PBS. To minimize the iron contribution, twenty four hours before the assay the standard medium was replaced with IFM⁵.

To evaluate the effect of L.a on iron absorbance, one hundred µl of overnight culture of the bacterial solution adjusted to 106 colony forming unit (cfu) per ml., were re-suspended in IFM, and added to each well. Then either 25, 50 or 75 µM iron as Fe(II)SO₄.7 H₂O (Sigma Aldrich) was added⁶,⁷.
To keep the volume in a constant level, an equivalent medium aliquot was removed as required before adding ferrous sulphate or bacteria., the plates containing iron and bacteria were incubated at 37°C with 5% CO₂. The incubation time was 24 hours as the bacteria reaches the plateau state of growth. Then the samples were removed by aspiration from the 12-well plates, and cells were rinsed with 1 ml of PBS. The cells were lysed by addition of 0.5 ml of the ionized water to each well and sonicated with a probe-type sonic dismembrator at lowest setting (<1 W output) for 15 s. The Caco-2 total protein level was determined in the lysates by the Bradford Assay(17). Ferritin in the lysates was determined by Spectro ferritin kit (Ramco laboratories, Inc. USA, S 22, ELISA (18). Ferritin concentration was normalized and expressed as ng/mg protein. Cells treated with iron alone served as a reference in each experiment. Each experiment was conducted in duplicate and repeated in three different occasion.

The effect of L.a on iron bioavailability by Caco-2 cells in the presence of phytic acid

The assay was performed exactly the same as the previous assay (2.4.1). The only extra step was the addition of 50 mmol l-1 of phytic acid as sodium phytate (Sigma P8810) to each well before the assay. Phytic acid concentration was selected two times greater than iron concentration, where the maximum decrease in iron absorbance could be observed(19).

In vivo assay

Animals and their diet

Twenty four male Sprague-Dawley rats weighing 125-150 g were obtained from the “Pasteur Institute of Iran” and were housed individually in stainless-steel and plastic cages at 25 ± 1°C, 55 ± 5% humidity in a 12-h light/dark cycle. They had free access to water and chow with 50 mg kg-1 (50ppm) iron content(20).

After 3 days of adaption, the animals were divided into four groups, each group including six with homogeneous weight. The rats were fed the basal diet and given 1 ml dispersion of test supplement by gastric gavage for 30 days. Group I received Fe, group II: Fe and phytic acid, group III: Fe and L.a, and finally, group IV: Fe, phytic acid or L.a. The Supplement dosage was 0.25 mg kg-1 body weight of Fe as Fe(II)SO₄ (Sigma Alderich), phytic acid/Fe molar ratio of 2:1 and 108 cfu ml-1 probiotic. All animals received humane care in compliance with the Guide for Care and use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH publication no. 85-23). All procedures of this study were approved by Institutional Animal Ethics Committee of Alborz University of Medical Sciences.

Experimental procedure

After 30 days of administration, blood sample was obtained by a cardiac puncture under superficial ether anesthesia, and then, animals were sacrificed. Serum iron was determined by a colorimetric method using ferrozine, pyridyl-phenyl sulfonic acid triazine at 560 nm in a Beckman DU 640 spectrophotometer(21).

Statistical analysis

All results were reported as mean ± standard deviation. The statistical significance of differences among groups was assessed by T-test and one-way ANOVA and followed by Tukey’s test, to identify the differences between treated groups and control. using SPSS software package (Version 16.0: Chicago, IL). A value of P<0.05 was considered as significant. All experiments were repeated at least three times.

Results

In vitro assay

The effect of L.a on ferritin formation in the presence of iron

The results showed that the phytase activity of L. acidophilus probiotic bacteria was 5.4mU.L.

The effect of L.a on ferritin formation in the presence of iron and phytic acid

The addition of bacteria to the cell line in the presence of phytate improved iron absorbance, when compared to control (iron and phytate). The effect of bacteria was associated to the phytate/iron concentration ratio. When probiotic bacteria was added to the mixture of 50 µmol l-1 of phytate and 25 µmol l-1 of iron, ferritin formation was significantly increased approximately 9 times more than what was observed in the control experiment (P<0.001) (fig. 1).
Bacterial addition to the wells, containing 75 µmol l-1 of iron and phytic acid significantly (P<0.05) enhanced ferritin formation by an extent of two times (Fig. 2).

In vivo assay

As we expected, phytic acid decrease iron absorption by 30% (p< 0.005). The addition of probiotic bacteria to the mixture of phytic acid and Fe, improved iron absorption more than 49% compared to the group II that received Fe+ phytate. This increase was significant (p< 0.005).

However, probiotic bacteria had no significant effect on iron absorption in the absence of phytic acid (Table 1).

Discussion

Probiotics are being emerged as functional foods and are of significant interest in the field of nutrition. In this context, specific health benefits of probiotics are under investigation and documentation, including improvements in selenium and zinc bioavailability. This research was designed based on the previous studies on the effectiveness of fermented food in improving blood iron. However, the ability of probiotic bacteria to improve iron bioavailability still remains to be elucidated.

The major findings of this study were as follow: 1) Caco-2 cell is a reliable model for the investigation of iron bioavailability in the presence of probiotic bacteria, 2) lactobacillus acidophilus improved iron bioavailability in the presence of phytic acid when added to Caco-2 cells, and 3) there was an strong effect of probiotic bacteria on improving of iron bioavailability in diet with high content of phytic acid in rat.

To study iron absorbance in vitro, the Caco-2 cell culture model was used. This technique is based on the solubility and absorption of iron. Various authors have used this model for studying iron bioavailability in the presence of iron enhancers and inhibitors. They stated that the results of in vitro studies were comparable with in vivo experiments.

LAB is the only microorganism that does not require iron for its growth neither ferritin nor other storage forms of iron have been reported in these bacteria. Thus, ferritin formation in the Caco-2 cell line in the presence of lactic acid bacteria, would not interfere with ferritin measurements.

After optimization the system, we preliminary observed that adding the bacteria to the medium decreased ferritin formation. This finding is in agreement with the Lin and Yen (1999) study. They explained that iron depletion may be caused by the iron chelating ability of the LAB; a characteristic that can account for their antioxidant activity.

In the present work, the inhibitory effect of phytic acid with iron absorption was measured. As it was expected, phytic acid decreased ferritin formation in the cell line. As well, the reduction of ferritin formation in the presence of phytic acid has

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Table 1: Serum iron concentration in experimental groups.

<table>
<thead>
<tr>
<th>Animal groups</th>
<th>Serum Fe (µg dl-1)</th>
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</thead>
<tbody>
<tr>
<td>Group I (Fe)</td>
<td>105.75*</td>
</tr>
<tr>
<td>Group II (Fe+ phytic acid)</td>
<td>79.7</td>
</tr>
<tr>
<td>Group III (Fe+ probiotic)</td>
<td>114.00*</td>
</tr>
<tr>
<td>Group IV (Fe+ phytic acid+ probiotic)</td>
<td>118.50**</td>
</tr>
</tbody>
</table>
been demonstrated by other researchers (33-35). Phytic acid is an iron inhibitor, being abundant in cereal, that strongly inhibit iron absorption to binding to iron and making insoluble compounds, which can not be accessed by intestinal cells (33-35).

Our experiments showed that the tested probiotic strain had phytase activity. The phytases are a group of enzymes that can degrade phytic acid. Phytase activity of some LABs was reported previously by others, but none of them were used as probiotics (35-38).

Some bacteria that have phytase enzymes can decrease the phytic acid content of food substrate. Food fermentation can degrade the phytate and increas iron absorption (40).

Finally, bacteria plus phytic acid were added to three different iron concentrations. Effectiveness of La observed better when was added to iron plus phytic acid. This effect may be the result of phytase activity of the isolate.

The correlation between phytase activity of lactobacillus bacteria and bifidobacteria on iron bioavailability improvement had been reported previously by Afify et al. (39, 40). They used an in vitro digestion/solubility assay to measure iron bioaccessibility from three varieties of fermented white sorghum (40).

Our study showed that probiotic bacteria strongly increase iron absorption from the diet inreached with phytic acid in rats. This could be explained by the phytase activity of the selected probiotic bacteria for the assay.

The effect of prebiotics on iron absorbance has been previously reported (11, 12). Prebiotics are non-digestible fibers such as inulin that specially increase the number and activity of probiotics in the gut. Freitas et al evaluated the effect of different prebiotics on the iron absorption, caecal weight and caecal pH in rats. They found that prebiotics significantly increased caecal weight and intestinal iron absorption. They explained that iron bioavailability was increased because of the decrease in pH accompanied by the production of acid by lactic acid bacteria in the gut. They also suggested that this effect could be related to the upregulation of the genes, which could increase iron absorption in the intestine (41).

In another study, young women with iron deficiency had low level of lactobacilli in the their faeces (43).

In conclusion, the optimized system examined in this study can be used to study the iron bioavail-

ability in the presence of probiotic bacteria. It seems that the effectiveness of probiotic bacteria is mainly on meals with high phytic acid, probably due to bacterial phytase activity. In future studies, the ideal balance between iron and probiotic numbers should be investigated. The other possible mechanisms by which probiotic bacteria affect iron absorption in vivo/ in vitro to be investigated.

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