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Iron Bioavailability and Pro- and Prebiotics

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Abstract

Iron deficiency is one of the most frequent micronutrient deficiencies around the world. Low iron bioavailability simultaneously with a high iron requirement is a high risk factor for developing iron deficiency. Probiotics are microorganisms that confer a health benefit on the host. Prebiotics are oligosaccharides which provide a health benefit on the host due to a positive modulation of the microflora in the gut. The aim of this paper is to evaluate if there is a connection between pro- and/or prebiotics and iron bioavailability. A literature review of studies published within the last ten years was done together with information from the organizations WHO, FAO and EFSA.

In the literature search, only four, published, human studies investigating the correlation between probiotics and iron bioavailability was found. Since the properties of probiotics always are strain specific studies done on different strains cannot be summarized in one conclusion. *Lactobacillus plantarum* 299v is a probiotic strain proposed to give enhanced iron absorption. An improvement of iron absorption was observed when the strain was added to a phytate rich meal. Trials with longer intervention periods are needed to assess the strain's impact on iron absorption. Addition of *Lactobacillus acidophilus* to an iron fortified beverage did not improve iron status among children. Contrary, a decreased risk of developing iron deficiency anemia was observed when *Bifidobacterium lactis* HN019 and prebiotic oligosaccharides were added to an iron-fortified beverage. The positive outcome can be an act of the pro- or the prebiotic or a collaboration of both.

The hypothesis that prebiotics can improve iron bioavailability, can unfortunately not be proven since there are not many human studies done to support the theory. Nevertheless there are four plausible biological mechanisms behind the hypothesis. The fermentation products, the short chain fatty acids, SCFA can release iron from complexes by a lowering of the pH, furthermore SCFA can enlarge the absorption area by stimulate the epithelial cells to proliferate. Prebiotics can give a reducing environment where iron can be reduced to the more soluble ferrous form. Additionally prebiotics can give an up regulation of the genes encoding for iron transporters and receptors. Studies performed on pigs indicate a positive outcome of prebiotic inulin on iron status. Additional human studies are required to support this result.

Conclusion: The relationship between pro- and/or prebiotics and improved bioavailability of iron requires further human studies to be confirmed.

Keywords: Iron bioavailability, probiotic, prebiotic, anemia.

Sammanfattning

Järnbrist är en av de vanligaste mikronäringsbristerna i världen. Låg biotillgänglighet av järn i samband med högt järnbehov är en hög riskfaktor för att utveckla järnbrist. Probiotika är mikroorganismer som ger en hälsofördel hos värden. Prebiotika är oligosackarider som ger en hälsofördel hos värden genom att ge en positiv förändring av tarmfloran. Syftet med denna uppsats är att utröna om det finns ett samband mellan pro- och/eller prebiotika och biotillgängligheten av järn. En litteraturstudie av studier publicerade de senaste tio åren har utförts tillsammans med information från organisationer som WHO, FAO och EFSA.

Litteratursökningen gav endast fyra, publicerade, humanstudier som utreder sambandet mellan probiotika och järnets biotillgänlighet. Eftersom probiotiska egenskaper alltid är stamspecifika så kan studier gjorda på olika bakteriestammar inte sammanfattas i en slutsats. *Lactobacillus plantarum* 299v är en probiotisk stam föreslagen att ge en ökning av järnabsorption. En förbättrad järnabsorption observerades då stammen tillsattes till en fytinsyrarik måltid. Försök med längre interventioner behövs för att kunna fastställa stammens påverkan på järnabsorption. Tillsatts av *Lactobacillus acidophilus* till en järnberikad dryck förbättrade inte järnstatusen hos barn. Däremot kunde en minskad risk för järnbrist anemi observeras då *Bifidobacterium lactis* HN019 och prebiotiska oligosackarider tillsattes till en järnberikad dryck. Den positiva effekten kan vara ett resultat av pro- eller prebiotika eller ett samarbete av båda.

Hypotesen att prebiotika skulle förbättra järnets biotillgänglighet har tyvärr inte kunnat styrkas av humanstudier, då för få har genomförts. Dock finns fyra möjliga biologiska mekanismer bakom hypotesen. Fermenteringsprodukterna av prebiotika, korta fett syror (SCFA) kan frigöra järn från komplex genom att minska pH. Därtill kan SCFA förstora absorptions ytan genom att stimulera prolifiering av epitelceller. Prebiotika kan ge en reducerande miljö som gör att järn kan reduceras till den mera lösliga Fe^{2+}-formen. Dessutom kan prebiotika ge upphov till en uppreglering av generna som kodar för järnets transportörer och receptorer. Studier gjorda på gris visar ett positivt samband mellan prebiotisk inulin och järnstatus. Ytterligare studier utförda på människor krävs för att styrka detta resultat.

Slutsats: Sambandet mellan pro- och/eller prebiotika och ökad biotillgänglighet av järn kräver flera studier utförda på människor för att kunna konstateras.

Nyckelord: Järn biotillgänglighet, probiotika, prebiotika, anemi.

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Introduction

Aim

Iron deficiency is one of the most common micronutrient deficiencies around the world. According to WHO 3 billion people are suffering from iron deficiency and 1 billion people from iron dependent anemia. Dietary modifications can be a method to enhance the bioavailability of iron and thus improve iron status worldwide. Pro- and prebiotics could be an efficient and innovative tool to improve the bioavailability of iron. The aim of this paper is to evaluate studies done on pro- and prebiotics and their impact on the bioavailability of iron.

Probiotics, definition

The World Health Organization (WHO) and Food and Agricultural Organization of the United Nations (FAO) have together stated a definition of probiotics. Probiotics are "Live microorganisms which when administrated in adequate amounts confer a health benefit on the host". WHO and FAO have some guidelines for probiotics. A food product has to contain a sufficient amount of microorganisms that also are alive when the product is supposed to be consumed, to be called probiotic. Also the microorganism should be able to survive the tough environment in the stomach. Beneficial probiotic properties are strain specific. Human studies should always be the basis of the probiotic health benefit. (FAO, 2006)

Prebiotics, definition

The definition of prebiotics, according to FAO is: "A prebiotic is a non-viable food component that confers a health benefit on the host associated with modulation of the microbiota". A prebiotic is always a compound, never an organism or a drug. The prebiotic should give a measurable health benefit, but not by absorption into the bloodstream. Furthermore a prebiotic should give rise to a positive modulation of the microbiota in the host. (FAO, 2007)

Material and Methods

A literature review was done to evaluate if there is a connection between pro- and prebiotics and iron bioavailability. Databases used for information retrieval were Scopus, Pubmed and Web of Knowledge. The search queries were: "iron AND bioavailability AND probiotic*" or "iron AND bioavailability AND prebiotic*". The search was restricted to human studies. Due to the small number of results for prebiotics some studies performed on pigs were included. Pigs are a good model animal for nutrition studies since the gastrointestinal tract resembles the one of humans (Patterson, 2008). The studies used in this mini-review are at maximum ten years old. For background information the book *Essentials of Human Nutrition* has been used. Webpages of reputable organizations like WHO, FAO and European Food Safety Authority (EFSA) have been utilized, together with other publications from these organizations.

Iron

The function of iron in the body

Iron is a micronutrient with several crucial functions in the human body. First of all iron plays a vital role of the oxygen transport in the body as it is a part of hemoglobin. The transport of oxygen is performed by the protein hemoglobin that can be found within the erythrocytes. Iron can also be found in the oxygen storage protein in the muscles, myoglobin. Within cells, iron is a transport medium for electrons. Iron also takes part in various enzyme systems in the body. The cytochromes are enzymes containing iron that are active in the oxidative metabolism where they transfer energy within the cells. Enzymes containing iron play an important part in body functions such as the synthesis of steroid hormones and detoxification of harmful substances in the liver. (FAO and WHO, 2004)

Iron absorption

The human body contains 4 g of iron. Most of the iron is stored as hemoglobin but about 1g is also stored as ferritin or hemosiderin, mainly in the liver. Excess iron can be hazardous for humans since iron can cause free radicals. In humans there is no regulation of the excretion of iron; therefore the absorption mechanism is very important. (Han, 2011)

In our diet two different forms of iron are available, heme iron and non-heme iron. Heme iron derives from the animal kingdom and is more bioavailable than the non-heme iron. There are many dietary components that may enhance or inhibit iron bioavailability, presented in *Table 1*. Notable is that non-heme iron is more affected by the composition of the diet than heme iron. In a western diet 2/3 of the absorbed iron is heme iron, even though 90% of the dietary iron is non-heme iron. (FAO and WHO, 2004)

Table 1. Dietary compounds that enhance or inhibit absorption of non-heme and heme iron. Based on information from FAO and WHO (2004).

Food Components	Non-heme iron	Heme iron
Citric acid	+	
Ascorbic acid	+	
Amino acids (eg. cysteine)	+	
Meat, fish	+	+
Calcium	-	-
Phenolic compounds, like in tea and coffee	-	
Phytates	-	

+ = Enhancing absorption, - = Inhibiting absorption

Factors enhancing the bioavailability of non-heme iron are ascorbic acid, citric acid and some amino acids. Citric acid is chelating the iron so it is kept in solution, which increases the absorption. Ferrous (Fe^{2+}) iron is more soluble and absorbable than ferric (Fe^{3+}) iron. By reducing the ferric iron to ferrous iron, ascorbic acid and amino acids promote the iron uptake. Several inhibiting
factors have been discovered, including phytate, polyphenols and tannic acid. The iron is then kept unavailable for the absorptive transporters by being tightly bound to a complex either in the lumen of the gut or inside of the enterocyte. (Han, 2011)

The major iron absorption takes place in the duodenum and upper jejunum (MacPhail, 2002). The apical uptake of non-heme iron and heme iron is different (*Figure 1*). When reaching the gastrointestinal tract most of the non-heme iron is in ferric form. To be able to be absorbed into the intestinal enterocyte it has to be reduced to ferrous form. This can be done either by dietary compounds like ascorbic acid or by the brush border membrane ferrireductase, most likely the enzyme duodenal cytochrome b (Dcytb). If the body is iron deficient or in a hypoxic condition the enzyme is expressed to a larger extent. When the iron is in ferrous state, it is transported via an apical iron transporter, divalent metal transporter (DMT1) into the enterocyte. For efficient transport the environment has to be acidic since DMT1 is a proton symporter. Heme iron must be released from dietary hemoglobin or myoglobin before it can be transferred to the enterocytes. The proteases of the stomach and small intestine are performing this break down. Heme iron is soluble and can cross the brush border membrane of the enterocyte. There is also a specific transporter for heme iron across the apical surface of the enterocytes. Heme carrier protein (HCP1) is one protein promoting heme iron uptake in duodenal enterocytes. After entering the enterocyte heme oxygenase (HO) oxidize the heme iron into ferrous iron. (Han, 2011)

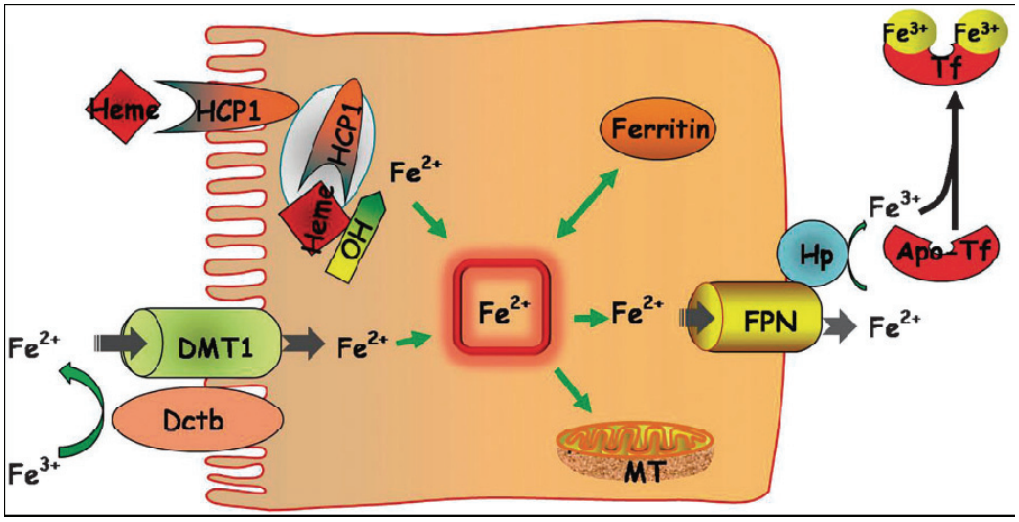

Figure 1. Non-heme ferric (Fe^{3+}) iron is reduced to ferrous (Fe^{2+}) iron by a ferrireductase, duodenal cytochrome b (Dcytb) or by dietary factors like ascorbic acid. The ferrous iron is then transferred across the enterocytes brush border via an apical transporter divalent metal transporter (DMT1). Heme iron has been released from the hemoglobin or myoglobin structure when it reaches the duodenum. It is now soluble and can cross the membrane, but there is also a promoting carrier protein, heme carrier protein (HCP1) that can transfer the iron into the enterocyte. Intracellular the iron can be transported to the mitochondria, to iron binding proteins or to the basolateral surface. The iron exporter ferroportin (FPN) transfers the iron across the basolateral surface. Before the iron is bound to transferrin it is oxidized by hephaestin (Hp). The iron is taken into the circulation with the transfer protein, Transferrin (Tf). Figure from: Han O. (2011). Molecular mechanism of intestinal iron absorption, Metallomics, 3, p.104. – http://dx.doi.org/10.1039/c0mt00043d
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Intracellular distribution of the iron will occur after transport into the enterocyte, most likely the mechanism is the same for non-heme and heme iron. The iron can face three different outcomes depending on the iron status of the body. It can be transported to the mitochondria for biosynthesis of heme and iron-sulfur clusters or it can be transported to ferritin for storage. The iron can also be transported to the basolateral surface. If the body is in need of iron it is rapidly

transferred to the circulation. Iron exporter ferroportin (FPN) is mediating the transfer across the basolateral membrane. Before the iron is bound to transferrin it is oxidized by hephaestin (Hp). Transferrin is a transfer protein responsible for the transport of iron in blood plasma. (Han, 2011)

Iron recommendations

Iron is not excreted via the urine or in the intestine to any significant extent. Hence the basal iron losses are low, estimated to be 14 µg/kg body weight and day for adults. (FAO and WHO, 2004)

Since the bioavailability of iron can vary from under 5% to over 15% depending on the composition of the diet, the recommendations are set to different values for different diets. In developing countries the diets are low in animal food sources and high in phytates and phenols from vegetables and cereals. Therefore diets in developing countries are estimated to contain 5-10% bioavailable iron. In a typical western-type of diet the estimate of bioavailability of iron is between 12-15%. The recommendations given by WHO are given for different diet compositions: 5, 10, 12 and 15% iron bioavailability. (FAO and WHO, 2004) The Nordic nutrient recommendations (NNR) are markedly differing from the recommendations given by WHO for young girls as demonstrated in *Table 2*. The different recommendations are due to different strategies for recommendations. The recommendations for girls in the age of 10-17 years given by WHO are set to cover the 95th percentile of the population, while NNR is covering only the 90th percentile of the population in their recommendation. The recommendations for the other age groups are set to cover the 95th percentile of the population for both NNR and WHO. (NNR, 2004)

Table 2. Recommendations of daily intake of iron according to NNR and WHO for different age groups.

	Recommended daily intake of iron (mg/day) for a dietary iron bioavailability of		
Group	NNR, 15 %	WHO, 15%	WHO, 10%
Children, 0.5-9 y	8-9	4-6	5-9
Boys 10-17 y	9 -11	9-13	14-19
Girls 10-17 y	11-15[a]	9-22	14-33
Women, fertile	15	20	29
Women, postmenopausal	9	7,5	11
Men	9	9	14

[a] Covers the 90th percentile of the population.

NNR and WHO do not have any special recommendations for pregnant women. The iron requirement is not only depending on the dietary iron bioavailability but also on the iron status of the pregnant women. It is recommended to have a good iron status in the beginning of the pregnancy; a body store of 500 mg iron should have a positive outcome for the iron status during the whole pregnancy. Newborn babies will get their required iron supply from breast milk. Although human milk contains a low concentration of iron, the bioavailability is high. Breastfed infants are most unlikely to develop iron deficiency, hence there is no recommendation for children up to the age of six months. (NNR, 2004)

Iron deficiency

Iron deficiency is a common nutritional problem in both developing countries and in industrial countries. According to WHO anemia is a health issue that afflicts over 2 billion people worldwide. The causes of anemia are often coexisting, but the biggest reason for emitting anemia is dietary iron deficiency, 50% of the cases of anemia have their origin in dietary iron deficiency. Anemia can also be caused by other factors such as infectious diseases like Malaria and HIV, genetic disorders affecting the red blood cells, but also be a result of micronutrient deficiency of folate, vitamin B12 or vitamin A. (WHO, 2008)

When iron deficiency is developing, the body's iron stores are reduced which is reflected in the decrease of serum ferritin concentration. Later on in the development stage the tissues develop iron deficiency which can be seen in a higher concentration of transferrin in the blood. At last the hemoglobin levels are decreased. (NNR, 2004)

There are three important indicators of iron status (Biesalski & Erhardt, 2007). The most common way to measure iron status of an individual is to measure the hemoglobin (Hb) content of the blood. Hemoglobin measurements are not the best way to determine anemia since there are many causes of anemia other than iron deficiency. Furthermore, patients with a high Hb baseline have to lose 20-30% of their body store of iron before they are counted as anemic (Zimmermann, 2008). Serum ferritin is another common indicator of iron status since it is directly proportional to the body stores of iron in healthy individuals. As mentioned earlier, the first stage of iron deficiency gives declined serum ferritin as the iron stores are getting depleted. The parameter serum ferritin has a major weakness since it is influenced by many factors. Infection and inflammation will increase serum ferritin since it is an acute phase protein. Hence serum ferritin is not an applicable indicator of iron status in areas where infections are common (e.g. in developing countries). Soluble Transferrin Receptor (sTfR) is an indicator suitable when infections are occurring, since it is not affected to the same extent as serum ferritin. Still the assessment of iron status with sTfR is expensive. The most accurate way to determine iron status is to combine analysis of Hb, serum ferritin and sTfR, together with analysis of parameters of infections. The most common acute phase infection protein used for detection of infection is CRP (C-reactive protein). (Biesalski & Erhardt, 2007) (WHO, 2007)

In iron deficiency interventions the most accurate parameter to use is serum ferritin together with hemoglobin (WHO, 2007). If the hemoglobin value
increases sooner than serum ferritin the cause of anemia is most likely iron deficiency. Contrary, when serum ferritin values are increasing more rapidly than hemoglobin the causes of anemia is most likely not iron deficiency.

Iron deficiency is defined as a hemoglobin concentration below the optimum value in an individual. Iron deficiency is the stage when the cellular iron storage used for normal metabolic and physiological functions is fully utilized. At this point the serum ferritin level is below 15 µg/l for adults. For children a serum ferritin level below 12 µg/l is defined as iron deficiency. The definition for iron deficiency anemia is a hemoglobin concentration below the 95th percentile of the distribution of hemoglobin in a population. For women a hemoglobin level <120 g/l is considered as an anemic value and for men the value is <130 g/l. In *Table 3* the cut-off values for anemia for different age and gender groups are presented. (WHO, 2001)

Table 3. Hemoglobin levels for assessment of anemia for different age and gender groups (WHO, 2001).

Group	Hemoglobin (g/l)
Children, 6 - 59 months	<110
Children, 5 - 11 years	<115
Children, 12 -14 years	<120
Non-pregnant women [a]	<120
Pregnant women	<110
Men[a]	<130

[a] >15 years of age.

All groups within a population can develop iron deficiency, but since iron is excreted to a small extent people without heavy blood losses are at a low risk of iron deficiency. Risk factors for developing iron deficiency are low iron intake, poor absorption due to a diet with a high concentration of phytate and phenolic compounds and periods of life when iron requirements are higher than usual. Hence risk groups for iron deficiency are infants, children, adolescents and women of childbearing age. Since the weaning period for infants requires a high iron intake related to the energy intake this stage is a critical period for both child and mother. In most developed countries an increased knowledge and information distribution together with iron supplementation to risk groups has resulted in a better iron status. Nevertheless, some groups (e.g. menstruating women and adolescents of both sexes) in developed countries still suffer of iron deficiency. These groups have increased iron requirements, since the menstruating women are having additional iron losses and adolescents are in a greater need of iron for a normal development. In developing countries iron deficiency is still a severe public health concern. Infants are the major concern since the iron status affects important development stages of the brain. (FAO and WHO, 2004)

The most alarming health consequences of iron deficiency are the increased risk of maternal and infant death. Inadequate iron nutrition can result in abnormal development of both physical and cognitive organs for infants and children. For adults the decreased working capacity is a negative consequence,
both for the individual and for the society. (WHO, 2001)

Iron overload and toxicity

Iron is a micronutrient that in excess consumption can have a hazardous outcome. Since iron status is almost exclusively regulated via absorption it is important to remember that an excess intake can be harmful. A high intake >30 mg/day can give serum ferritin levels over 300 µg/l for men and 200 µg/l for women, which are regarded as unhealthy high iron stores. Iron overload in humans can give acute symptoms like nausea, vomiting, heartburn and constipation. Pharmaceutical iron supplements can cause mucosal erosion which can lead to severe damage of the liver, pancreas and the kidney. Iron can be lethal if ingested at levels above 180-300 mg/kg body weight. Hemochromatosis is a chronic iron overload that usually is inherited. Iron is accumulated and stored in the liver. Some individuals that ingested therapeutic amounts of iron for decades have generated secondary hemochromatosis. (NNR, 2004)

Methods to determine the bioavailability of iron

One definition of iron bioavailability is: "a measure of the proportion of the total in a food or diet that is digested, absorbed and metabolized by normal pathways" (Fairweather-Tait, 1987). To study the bioavailability four different techniques can be useful. There are some arithmetic models that can be used to calculate iron bioavailability based on prediction of the impact of enhancing and inhibiting dietary factors. Since the mathematical models are based on *in vitro* data and the results only are predictions, the reliance of these results is not sufficient to build a hypothesis for human iron bioavailability. Iron solubility studies are done *in vitro* to study how soluble iron will be after passing the stomach. The studying of iron solubility is not a very powerful method since it only measure the first part of iron bioavailability. Iron absorption studies can be done in several ways. The traditional method is to use native iron and study the chemical balance in humans. The apparent iron absorption is calculated by subtracting fecal iron from iron intake. The chemical balance can be studied with the help of radioiron or stable isotopes to give a more accurate result. Endpoint studies will give the best indication of bioavailability since all three parts of the expression is represented; solubility, absorption and incorporation. Commonly the hemoglobin levels before start and at the end of the study are measured. (Wienk, 1999)

The Hemoglobin Repletion Efficiency (HRE) can be used to measure the total increase in hemoglobin iron against the total iron intake. HRE is calculated by subtracting the initial total body hemoglobin iron from the final value and divide it with the total iron intake. For studying iron bioavailability in humans, the isotope studies are the most appropriate. A stable isotope is usually used. By comparing the changing isotopic ratio with the natural iron concentration in a specific tissue or blood sample the absorption can be estimated. (Patterson et al, 2008)

Probiotics

Micro-organisms such as bacteria and yeasts can be added to a food product to yield a positive outcome for the health-being of humans. These micro-organisms are called probiotics. Most of the probiotics are bacteria belonging to
the genera *Lactobacillus* and *Bifidobacterium.* Health benefits associated with probiotics are alleviation of symptoms due to infections in the gastrointestinal tract including diarrhea and irritable bowel syndrome. Furthermore probiotics can be used to relief the symptoms of allergy and improve the host's immunity. (WHO and FAO, 2001)

Probiotics might be an innovative tool to enhance the bioavailability of iron. Human studies have been performed to investigate the relationship between probiotics and iron status. However the quantity of human studies is low. Since probiotic properties are strain specific it would not be possible to say that all probiotics can increase the bioavailability of iron. Therefore the studies done on different strains are presented separately in this paper. All human studies found on the search query presented in the methods section are discussed in the text and also summarized in *Table 4*.

Lactobacillus plantarum and non-heme iron absorption

Lactobacillus plantarum 299v is a probiotic strain hypothesized to have a positive outcome for iron absorption (Bering *et al*, 2006 and 2007). According to the authors it has previously been shown that lactic-acid fermented vegetables and cereals can increase the iron absorption. The

plausible mechanism has previously been described as an effect of the low pH and organic acids. The low pH can prevent the formation of complexes with low solubility and also activate phytases. The organic acids can chelate with iron and keep it in solution so it is more absorbable. Organic acids may also delay the gastric emptying that increases the iron exposure to the proximal intestinal epithelium which might lead to increased iron absorption.

To be able to determine if live *L. plantarum* 299v in a meal would give a different result on iron absorption than its fermentation products, a cross-over trial with four different meals was done. Subjects of the study were young healthy women with low iron status, but not anemic. The study design was set to examine if live microorganism would give difference in iron absorption compared to a meal with adjusted pH or added organic acids. The test meal, (A) contained fermented oat gruel with added *L. plantarum* 299v (10^9 CFU/g). A total of 3 controls were used, (B) pasteurized, fermented oat gruel, (C) non-fermented oat gruel with pH-adjustment and (D) non-fermented oat gruel with organic acids added. Significantly higher iron absorption was observed from the test gruel with *L. plantarum* 299v compared to the control meals (P<0,0001). However, analysis of the test and control meals revealed a difference in the organic acid concentration. The difference in organic acid concentration could affect the result of iron absorption. The smallest difference in lactic acid concentration was observed between meal A and D where the concentration was 19% higher in test meal A, however the iron absorption was 50 % larger in the meal containing live *L. plantarum* 299v. (Bering *et al,* 2006)

Examination of the importance of the organic acid concentration resulted in a new trial (Bering et al, 2007). The study resembles the previous study with same type of subjects, randomized cross-over design and same type of meals. Only two test meals were used, heat-inactivated lactic acid-fermented oat meal with or without added viable, lyophilized *L. plantarum* 299v. The heat-inactivation made it possible to keep the organic acid content stable. No significant difference on iron absorption could be observed. The reason for no observed effect on the iron absorption in the second study was discussed by the authors. One explanation could be that the lyophilized bacteria was not in a sufficient amount when reaching the proximal small intestine and thus too weak to colonize. (Bering *et al*, 2007)

An interesting point of view would have been to examine whether a colonization of the strain had occurred in the intestine. Unfortunately this was not studied. Furthermore the intervention periods of both studies are short. The first study was done in two periods for four days each, while the second study was done in two periods with two days in each period. A longer intervention period could have affected the iron absorption positively and perhaps strengthen the result of the first study.

Within the European Union all foods with health claims have to be authorized by The European Food Safety Authority (EFSA) according to regulation EC No 1924/2006. EFSA has rejected an application from the company Probi for the health claim "*Lactobacillus plantarum* 299v (DSM9843) improves iron absorption". According to the scientific panel the cause and effect relationship is not sufficient enough. Other studies on *L. plantarum* than the two presented in this paper have been done, but are not published, as can be seen in the application sent to the European Food Safety Authority (EFSA, 2009). When trying to contact the company Probi to get the results of the studies, the company refused to share the results since the material contains confidential material.

Since EFSA did not accept the health claim it could be easy to assume that there is no effect of the strain on iron absorption. Desirable would be to continue to study the relationship between the probiotic strain *L. plantarum* 299v and iron absorption. The question if there will be any

companies ready to make more studies on the strain raises. Human studies are expensive and require a lot of time. Food companies might be willing in doing research but are naturally interested in being able to use the results in promotion of their food products. Research should be done by independent researchers with independent funding. Hopefully the future will give studies performed in this perspective that evaluate the link between *L. plantarum* 299v and iron absorption.

Probiotics, iron-fortified beverages and iron status of children

Fortification of food products is an efficient way to defeat iron deficiency anemia. Preschool children are a group for whom an improved iron status is extra relevant, since a low iron status influences physical and cognitive performance in a negative way (WHO, 2008).

The effect of *Lactobacillus acidophilus* on iron-fortified fermented milk was investigated in a study by Silva *et al* (2008). The study was done on preschool children that usually had a diet with low-bioavailable iron. A fermented milk beverage was provided to 190 children in the age 2-5 years old for 101 days. The beverages were fortified with an iron-amino acid chelate and to the test product 10^8 CFU *L. acidophilus*/ml was added, to the control group no extra bacteria was added. After the intervention Hb values in test and control group did not differ. The authors of the study are interpreting the difference in the MCHC (Mean Cell Hemoglobin Concentration) as an increased bioavailability of iron in the probiotic group (Silva *et al*, 2008). MCHC is used to assess the average Hb concentration in red blood cells (Fischback & Dunning, 2009). Iron deficiency is monitored as a decrease in MCHC. Silva *et al* (2008) are concluding the significant higher MCHC value for the test group as a higher red blood cell status. The conclusion made by the authors is optimistic. A low MCHC can also be a result of anemia due to inflammation (WHO, 2008). In the control group an increase in the acute phase protein serum ferritin was observed. The significant difference in MCHC values between test and control group might be a result of inflammation in the control group. Unfortunately no indicators for infections or inflammation such as RCP were determined in the trial. The statement that iron status was improved by the probiotic strain should have resulted in a more significant difference in other parameters like Hb and serum ferritin between the test and control group. When comparing the before and after intervention values for all the subjects, both in test and control group, a decrease in Hb could be observed. The children stayed at the border to anemia, with a mean Hb of 116 g/l and 115 g/l for the probiotic and test group respectively. The fact that the children still were close to anemia after an intervention for 101 days with a product containing 3 mg iron per serving is a bit unexpected. This can be explained by a diet containing a high amount of iron inhibiting components such as phytates.

The result of a long-term (1y) study performed on a large number of children (n=624) did not result in any significant difference in individual iron status in a test group fed with a beverage containing the probiotic strain *Bifidobacterium lactis* HN019 and prebiotics compared to the control group (Sazawal *et al*, 2010). In both, the test and control group, the milk was fortified with nutrients to meet nutritional guidelines; hence both groups deceived 5.4 mg of iron per day with the provided drinks. The test product contained 10^7 CFU/day of *B lactis* HN019 and 2,4g/day of oligosaccharides. The study showed a 45% lower risk of developing iron deficiency anemia in the probiotic group than in the control group. The criteria for being anemic in the study was set to Hb <100 g/l and to be considered iron deficient 2 abnormal values of the following parameters had to be observed; serum ferritin <12 µg/l, serum transferrin >8.3 µg/ml, hematocrit ≤30% or zinc protoporphyrin ≥80 µg /mol heme. The four parameters are all considered to be good indicators of iron stores (WHO, 2007). Serum ferritin is reflecting iron stores, but has a major draw-back with the influence from infections. Serum transferrin is decreased when iron deficiency is

developing. Hematocrit relates to the content of red blood cells in the blood. The zinc protoporyhyrin indicates the lack of iron for red blood cell development. In case of iron deficiency or infection the zinc protopoyhyrin will increase (WHO, 2007). Since both serum ferritin and zinc protoporyhyrin are affected by infections it would have been favorable to have an indicator of infections in the trial. The positive outcome, a decreased risk of developing iron deficiency anemia, is a result of added pro-and prebiotics. It cannot be said if the effect is a consequence of the microorganism, the oligosaccharide or a collaboration of both, called synbiotics.

The importance of relevant parameters for measuring iron status becomes clear. If a trial is performed in a developing country infections are common. Since infections can influence several indicators (e.g. serum ferritin and zinc protoporyhyrin) used for iron status determination it is relevant to measure if an
infection is present in the study group. According to WHO the most accurate way to determine iron status is to combine analysis of Hb, serum ferritin and sTfR, together with analysis of parameters of infections (WHO, 2007).

Furthermore the length of the intervention period is essential. The intervention period should be set to be able to give an effect on the subjects of the trial. The selection of subjects should also be chosen after what kind of research is performed and to suit the thought target group. It is important to remember that subjects with adequate iron stores and iron sufficient subjects might respond differently to the same treatment.

Table 4. A summary of the studies performed on humans during the last decade investigating the relationship between probiotics and iron bioavailability.

Probiotic strain and dose	Subjects	Study design	Result
Lactobacillus plantarum 299v[1] 10^9 CFU/g = 10^{11}CFU/day[5]	24 women with low iron stores (serum ferritin 12-40 µg/l, Hb ≥ 110 g/l)	Randomized, double blinded cross-over trial. 4 test meals: (A) Fermented oat gruel with viable *L. plantarum* 299v (B) Pasteurized, fermented oat gruel (C) non-fermented oat gruel with pH-adjustment (D) non-fermented oat gruel with organic acids added.	The oat gruel with live *L. plantarum* 299v significantly increased the iron absorption.
Lactobacillus plantarum 299v[2] 10^9 CFU/g = 10^{11}CFU/day[5]	18 women with low iron stores (serum ferritin < 30 µg/l)	Randomized, double blinded cross-over trial. 2 test meals: (A) Heat-inactivated lactic acid-fermented oat gruel with added viable, lyophilized *L. plantarum* 299v. (B) Heat-inactivated lactic acid-fermented oat gruel without added probiotic strain.	No significant difference in iron absorption could be observed from the two test meals.
Lactobacillus acidophilus[3] 10^8 CFU/ml = 8 x 10^9 CFU/day[5]	190 children 2-5 years old	Parallel study design. 2 test beverages: Iron fortified milk beverage with added probiotic *L. acidophilus* (test) and without the strain (control).	No significant difference between test and control group in Hb or serum ferritin levels. Significant difference in MCHC[6] between test and control group.
Bifidobacterium lactis HN019[4] 10^7CFU/day (+ prebiotic oligosaccharides)	624 children 1-3 years old	Randomized, double blinded parallel trial with iron fortified milk beverage with added pro- and prebiotics (test) and the same beverage without added pro- and prebiotics (control).	No significant difference between test and control group in Hb or serum ferritin levels. Decreased risk for iron deficiency anemia

				in the probiotic group.

[1] Bering et al (2006); [2] Bering et al (2007); [3] Silva et al (2008); [4] Sazawal et al (2010)
[5] Since the dose given by Sazawal et al (2010) was a daily intake dose and the other studies presented a CFU/ml or g, the daily providing of probiotics for the other studies was calculated and presented in the table.
[6] MCHC = Mean Cell Hemoglobin Concentration

Lactobacilli, Bifidobacteria and pathogenic bacteria

Lactobacilli and Bifidobacteria are two genuses of non-pathogenic bacteria. Both genuses can be found naturally in the human gut, but are also used as probiotics. Bacterias belonging to the genus of Lactobacilli or Bifidobacteria do not require much iron for their growth. A microflora consisting of these bacteria might lead to more available iron for the host. Pathogenic bacteria have the adverse effect since they require a lot of iron for their function. Probiotics strains compete with pathogenic strains like clostridia. Thus a microflora with a large concentration of Lactobacilli and Bifidobacteria would be beneficial for humans with low iron status since the availability of iron is enhanced as a result of the knock-out of the pathogenic bacteria using the dietary iron. (Patterson et al 2008)

The question lingers, if this amount of iron is significant for people with iron deficiency? In any case, the probiotics strains belonging to the genuses mentioned above do not seem to be negative for the bioavailability of iron.

Prebiotics

Prebiotics are oligosaccharides that cannot be digested by humans but are fermented by the microflora in the human gut. Thereby prebiotics selectively stimulate the growth and activity of bacteria in the colon to confer a health benefit to the host. Enhanced mineral absorption is one potential health benefit that prebiotics could confer. The most common and mostly investigated prebiotic is inulin, a fructan naturally occurring in a range of plants like chicory root, Jerusalem artichoke and onion (Yeung et al, 2005). Poor iron bioavailability is common in developing countries were the diet is based on vegetables rich in phytates and phenols. Hence, dietary modification can be an efficient way to fight the problems with iron deficient anemia (WHO, 2001). Prebiotics such as inulin and fructooligosaccharides have been suggested to have an iron absorption increasing property (Yeung et al, 2005). Since no studies performed *in vivo*, on humans the last decade can be found, there are not sufficient evidence for the hypothesis; however there are some reasonable theories behind the mechanisms.

Several features belonging to prebiotics could explain an enhancement of bioavailability of iron. The fermentation products, the Short Chain Fatty Acids, SCFA can promote iron absorption by two actions. SCFA are acetic, propionic and butyric acids that make the environment in the colon more acidic. The lowering of the pH might enhance mineral absorption by releasing the iron from protein-complexes. Furthermore one of the fermentation products, propionate can form soluble complexes with iron and hence make the iron more absorbable. Additionally SCFA stimulates the

proliferation of epithelial cells. Thus the absorptive area in the colon is increased. Prebiotics can also produce an environment that is reducing, hence promoting iron uptake by the reduction of ferric iron to ferrous iron, which is more soluble by a factor of 10^{17}. An up-regulation of genes expressing transport proteins and receptors promoting iron absorption could also be the result of prebiotics. Iron absorption can be promoted by more expression of iron transfers like Dcytb or DMT-1, see *Figure 1.* Previously dietary fructooligosaccharides has showed enhanced calcium uptake due to increased expression of calbindin-D9k, a protein involved in intestinal calcium transport. (Yeung *et al*, 2005)

In the last decade no published, human studies investigating the relationship between prebiotics and iron bioavailability have been found. Earlier studies were discussed in a review by Yeung and others (2005). The studies done on human subjects, evaluating the connection between prebiotics and iron bioavailability have almost exclusively been done on men with a good iron status. The results of the studies did not give any significant difference between the prebiotic and control group. In both studies the vitamin C intake was high and the ascorbic acid might have masked the effect of prebiotics (Yeung *et al*, 2005). A more relevant study would be to investigate the impact of prebiotics on a meal with low iron bioavailability on a group with low iron stores.

Studies done on animals will always give results that are scanty. Still animal models can give us a better understanding in a topic and some plausible mechanisms for a hypothesis. When studying iron absorption and bioavailability rats are not a good laboratory animal. One of the reasons why studies done on rats not are valid for humans is that rats absorb ferric and ferrous iron to the same extent. The intestinal environment of rats differs from human's anatomy. Rats have phytase activity in their intestine and they can synthesize ascorbic acid. (Wienk et al, 1999)

Pigs have a gastrointestinal tract with similar physiology and function like the human gut (Patterson et al, 2008). Although the intestine of pigs is heavier and longer than the human intestine the transit time is almost the same. Additionally pigs have an enteric microbiota that resembles the one of humans. Pigs can therefore be a good experimental model for studying mechanisms applicable in human nutrition.

A diet supplemented with 4% of the prebiotic inulin increased the iron bioavailability in iron deficient pigs. Piglets fed with a diet supplemented with 4% inulin had a 15% higher Hb concentration, after a five week intervention, compared to piglets fed with a basal diet (Yasuda *et al*, 2006). One of the mechanisms for the increased bioavailability of iron could be an enhanced expression of iron transporters and receptors. Dietary inulin has shown the ability to affect the gene expression of transporters and receptors vital for iron bioavailability (Tako *et al*, 2008). The study was done on pigs and a 4% supplementation with inulin gave a significant difference in the mRNA expression in both the duodenum and colon. In the duodenum the gene expression of ferritin, DMT1, Dcytb, ferroportin and transferritin receptor (Tfr) was increased. In the colon, an inulin supplemented diet increased the gene expression of DMT1, Dcytb and ferritin when compared to the control diet. The iron transporters and receptors are all important in iron absorption as can be seen in *Figure 1.* An up regulation of the expression of encoding genes of all the before mentioned transporters and receptors can give rise to an increase of the bioavailability of iron.

Prebiotics pass the small intestine nearly intact and are fermented in the colon by the microflora. Hence prebiotics have been hypothesized to enhance iron bioavailability by increasing the colonic iron absorption. (Patterson et al, 2009) Iron absorption does mainly occur in the duodenum (MacPhail, 2002). To investigate if also the colonic absorption can contribute to the total intake studies on pigs have been done. By feeding anemic piglets with two different iron isotopes, one

fed orally and one infused to a cecal cannula, it is possible to measure total and colonic iron absorption, respectively. Two trials done in this manner show the same result; colon is not an important site for iron absorption. Furthermore, the studies showed that inulin did not affect colonic absorption (Patterson et al, 2009). Yeung and others (2005) are discussing the few measurements of colonic iron absorption done on humans in their review article. Previous studies show a central colonic absorption of ferrous chloride (9.3%) but only modest absorption of ferric chloride (0.5%). Some data from a trial with iron deficient dogs suggest that colon can be a significant site for iron absorption in iron deficient subjects (Yeung et al, 2005). Further human trials should be done to examine if the colon can be a significant absorption site for iron. Even if it cannot yet be proven that colonic iron absorption occurs, prebiotics might improve the iron status by a positive modulation of the resident microbiota in the small intestine. This was proven when inulin showed a favorable modulation of the microbiota in the jejunum and ileum in piglets. The natural microflora might have an influence on iron absorption by impacting the intestinal barrier. (Patterson et al, 2009)

The supplementation of 4% prebiotic inulin show a positive outcome on iron bioavailability in pigs (Yasuda *et al*, 2006; Tako *et al*, 2008). Still it is important to remember that pigs and humans might respond differently to prebiotics. Hence more studies performed on human subjects are required to establish the positive effect of prebiotics on iron bioavailability.

Conclusions

The results of the few studies done on probiotics and their impact on bioavailability of iron do not show a sufficient cause and effect relationship. The possible correlation between probiotics and improved bioavailability of iron should be strengthened by further human studies. Some data suggest an improvement of iron absorption when *Lactobacillus plantarum* 299v was added to a phytate rich meal. More studies with longer intervention periods should be done. An addition of *Lactobacillus acidophilus* to an iron fortified beverage did not improve iron status among children. The addition of *Bifidobacterium lactis* HN019 and prebiotic oligosaccharides to an iron fortified beverage decreased the risk of developing iron deficiency anemia in a group of children. The positive outcome can be an act of the pro- or the prebiotic or a collaboration of both.

Prebiotics do not have an effect on human iron bioavailability with today's burden of proof. Although it seems like prebiotic inulin increase the bioavailability of iron when administrated to pigs. Additional human studies are required to confirm the results from the animal trials.

The future research should be performed on humans and utilize adequate parameters of iron status to assess the impact of pro- and prebiotics. Parameters sufficient for the determination of iron status are a combined analysis of hemoglobin, serum ferritin, soluble transferrin receptor together with analysis of parameters of infection.

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