THE EFFECT OF *LACTOBACILLUS PLANTARUM* 299V AND LACTIC ACID ON IRON BIOAVAILABILITY

- *In vitro* and human studies

PhD thesis

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Frederiksberg, Denmark, 2006

The effect of *Lactobacillus plantarum* 299v and lactic acid on iron bioavailability – *In vitro* and human studies

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ISBN-10: 87-90505-24-7
ISBN-13: 97-88-7905-05-240
Printed by Samfundslitteratur Grafik, Dyrlægevej 5, 1870 Frederiksberg C, Denmark

PREFACE

The present thesis is based on work carried out at The Department of Human Nutrition, Centre for Advanced Food Studies, KVL, Denmark, with a part of the practical work carried out at BioCentrum, The Technical University of Denmark, and The Institute of Food Research, Norwich, UK, throughout the years 2001-2006, interrupted by two years of maternity leave.

The project was a part of the Øresund center contract 'Integrated systems for the development and control of characteristics in microorganisms for probiotics and food', in which the following partners participated: Skåne Dairy, Probi AB, SIK AB, Ideon Agro Food, and The University of Lund from Sweden, and Novozymes A/S, Schulstad A/S, Bioneer A/S, Danish Toxicology Center, and KVL from Denmark.

The PhD project was financed by the Ministry of Science, Technology and Innovation, Denmark, and Vinnova, the Swedish Agency for Innovation Systems. The exchange program at the Institute of Food Research, Norwich, UK, was financed by the Marie Curie Fellowship, an EU-funded mobility program.

Stine Bering
May 2006

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LIST OF PAPERS

The present PhD thesis is based on the work contained in the following three papers, referred to in the text by their roman numbers:

I. Bering S, Bukhave K, Henriksen M, Sandström B, Pariagh S, Fairweather-Tait SJ, Lund EK. Development of a three-tier *in vitro* system, using Caco-2 cells, to assess the effects of lactate on iron uptake and transport from rye bread following *in vitro* digestion. J Sci Food Agric 2006;86:2438-2444.

II. Bering S, Suchdev S, Sjøltov L, Berggren A, Tetens I, Bukhave K. A lactic acid-fermented oat gruel increases non-haem iron absorption from a phytate-rich meal in healthy women of childbearing age. Br J Nutr 2006;96: 80-85.

III. Bering S, Sjøltov L, Suchdev S, Berggren A, Alenfall J, Jensen M, Højgaard L, Tetens I, Bukhave K. Viable, lyophilized lactobacilli do not increase iron absorption from a lactic acid fermented meal in healthy young women, and no iron absorption occurs in the distal intestine (submitted).

SUMMARY

Iron deficiency remains one of the most important nutritional deficiencies and is a major health concern worldwide. Iron supplementation is efficient in treating iron deficiency but is associated with adverse side effects due to gastrointestinal distress and potential risks of oxidative damage. Alternative strategies for alleviating iron deficiency are food iron fortification or dietary modification to increase iron bioavailability. Lactic acid fermentation is a simple natural food preparation method that have gained new interest in the concept of probiotics, and lactic acid fermented foods have shown to significantly enhance iron absorption in humans, possibly by increasing the amount of enhancers and decreasing the amount of inhibitors. Furthermore, rat studies have been shown to increase iron absorption from both pre- and probiotics, with significant contributions to the absorption of iron in the colon.

The major aims of this project were to investigate the specific effects of a viable probiotic bacterium, *Lactobacillus plantarum* (*Lp*) 299v, and the fermentation products, mainly lactate, on nonheme iron absorption from phytate rich foods. This involved improvement of an *in vitro* digestion/Caco-2 cell model to measure iron dialyzability, uptake, and transport directly from an *in vitro* digested food matrix, thereby being able to determine the effect of lactate on iron bioavailability (Paper I). The *in vitro* study was followed by two randomized, double-blinded, single meal cross-over trials with healthy young women, to investigate the effect of the viable *Lp* 299v versus the organic acids in a lactic acid fermented oat gruel on nonheme iron absorption (Paper II), and the effect of viable lyophilized versus heat-inactivated *Lp* 299v in a fermented oat gruel on nonheme iron absorption, with elucidation of any potential iron absorption in the ileum and colon (Paper III).

In the 3-tier Caco-2 cell system the effect of lactate (0-200 mmol/L) on iron dialyzability, uptake and transport across the Caco-2 monolayer from a food

matrix of rye bread subjected to simulated peptic and pancreatic digests was investigated. The peptic digests were digested with pepsin at pH 2.0 and then increased to pH 5.5 before incubation and the pancreatic digests were peptic digests added pancreatin and bile at pH 6.5 and incubated directly in the model to simulate absorption in the most proximal duodenum and jejunum, respectively. Whereas lactate (200 mmol/L) increased the dialyzability of iron from 13 ± 2% to 24 ± 3% (mean ± SEM, n = 4, $P < 0.05$) in the peptic digests, iron uptake was in the region 39 ± 9 to 76 ± 11 pmol/mg protein, with no effect of lactate. In contrast, during the pancreatin digestion the dialyzability of iron decreased from 6.9 ± 0.2% to 2.0 ± 0.2% ($P < 0.001$), and the uptake decreased from 228 ± 30 to 8 ± 1 pmol/mg protein, and these two factors were positively linearly correlated ($R^2 = 0.41$, $P < 0.01$). Due to low dialyzability of ^{14}C-polyethylenglycol (PEG) in the pancreatic digests and compromised monolayers (2.4 ± 0.1%/(h × cm^2) transfer of PEG, n = 40), the transcellular iron transport was estimated for the peptic digests only, and only at 200 mmol/L lactate was there any significant transcellular transport of iron (180 ± 47 pmol/mg protein, $P < 0.05$), resulting in a positive correlation between iron dialyzability and total iron uptake from the peptic digests ($R^2 = 0.48$, $P < 0.01$). In conclusion, the 3-tier Caco-2 cell system showed the potential to obtain detailed insight into each step involved in iron transport across the monolayer from a food mixture, and the effect of lactate on iron uptake from a phytate rich food was associated mainly with iron bioaccessibility.

In human study I 24 healthy women aged (± SD) 25 ± 4 y with low iron status (serum ferritin < 40 μg/L) were served (A) a fermented oat gruel, (B) a heat-inactivated fermented oat gruel, (C) a pH-adjusted non-fermented oat gruel, and (D) a non-fermented oat gruel added organic acids. The meals were extrinsically labelled with ^{55}Fe or ^{59}Fe and consumed on 4 consecutive days, *e.g.* in the order ABBA or BAAB followed by CDDC or DCCD in a second period. Nonheme iron absorption was determined from isotope activities in blood samples. The fermented oat gruel increased iron absorption significantly ($P < 0.0001$) compared to the heat-inactivated and non-fermented oat gruels, which were similar in iron, phytate, polyphenol con-

tent, and pH, but varied in lactate concentration (43-110 μmol/g). The lactate concentration in the fermented oat gruel was 19% higher than in the heat-inactivated fermented oat gruel, whereas the iron absorption was increased by 50%. In the oat gruel with organic acids, the lactic acid concentration was 52% lower than in the heat-inactivated gruel, with no difference in iron absorption. The results indicate that iron absorption from a lactic acid fermented oat gruel is increased possibly due to a specific effect of the viable *Lp* 299v and not only an effect of organic acids.

In human study II 18 healthy women aged (± SD) 22 ± 3 y with low iron status (serum ferritin < 30 μg/L) were served a fermented oat gruel with (A) viable and (B) heat-inactivated *Lp* 299v. The oat gruels were extrinsically labeled with ^{59}Fe and served with 2 enterocoated capsules (containing ^{55}Fe(II) and ^{55}Fe(III), respectively) designed to disintegrate in the ileum. The meals were consumed on 2 consecutive days, *e.g.* in the order AA followed by BB in a second period. Nonheme iron absorption was determined by ^{59}Fe whole-body retention and isotope activities in blood samples. The concentrations of iron, lactate, phytate, polyphenols, and pH were similar in the two meals, and there was no difference in iron absorption from the meals with viable lyophilized and inactive *Lp* 299v (1.4% and 1.3%, respectively). Furthermore, no absorption of ^{55}Fe in the ileum and colon was observed.

In overall conclusions, the effect of lactic acid fermented meals with viable lactobacillus seems to be caused mainly by organic acids. The specific effect of the viable *Lp* 299v on iron absorption from a lactic acid fermented oat gruel in human study I was not documented with viable lyophilized lactobacillus in human study II. Furthermore, no absorption occurs in the distal part of the intestine from low iron bioavailability meals with probiotics in healthy young women with low iron status. This is in good agreement with the results from the *in vitro* cell studies showing a positive effect of lactate in the most proximal small intestine. Lactic acid fermentation of foods as a means of dietary modification was therefore confirmed to increase iron bioavailability from vegetables and cereals although the impact is relatively minor, and it was not possible to direct it to a specific effect of the lactobacillus.

SUMMARY IN DANISH

Jernmangel er en af de vigtigste ernæringsmæssige mangelsygdomme. Jernpræparater er effektive til behandling af jernmangel, men er også behæftet med gastrointestinale bivirkninger og potentiel risiko for oxidativ skade. Alternative strategier til behandling og forebyggelse af jernmangel er berigelse af fødevarer med jern eller en modificering af fødevarer, således at biotilgængeligheden af jern fra kosten øges. Mælkesyrefermentering er en simpel naturlig forarbejdningsmetode, som med udviklingen af probiotika har fået fornyet interesse, og det har vist sig at mælkesyrefermenterede fødevarer øger absorptionen af jern i mennesker, formodentlig ved at øge mængden af fremmere og mindske mængden af inhibitorer. Derudover har rottestudier vist en øget absorption af jern fra både pre- og probiotika, hvor colon har bidraget signifikant til absorptionen af jern.

Formålene med dette projekt var at undersøge effekten af en levende probiotisk bakterie, *Lactobacillus plantarum* (*Lp*) 299v samt fermenteringsprodukter herfra, hovedsagelig mælkesyre, på absorptionen af jern fra fytinsyreholdige fødevarer. Dette inkluderede forbedring af en *in vitro* fordøjelse/Caco-2 cellemodel til måling af både dialyserbarheden af jern, samt optag og transport over cellelaget direkte fra en *in vitro* fordøjet fødevarematrix, og dermed bedre bestemmelse af effekten af mælkesyre på biotilgængeligheden af jern (artikel I). *In vitro* studiet blev fulgt op af to randomiserede, dobbeltblindede, parrede enkeltmåltidsstudier med raske unge kvinder til bestemmelse af effekten af henholdsvis levende *Lp* 299v og organiske syrer i en mælkesyrefermenteret havrevælling på absorptionen af nonhæm jern (artikel II), samt effekten af henholdsvis levende lyofiliserede og varmeinaktiverede *Lp* 299v i en mælkesyrefermenteret havrevælling på absorptionen af nonhæm jern, ved samtidig bestemmelse af en potentiel absorption af jern i ileum og colon (artikel III).

Cellemodellen blev udviklet til et tre-lags Caco-2 cellesystemet, hvor effekten af mælkesyre (0-200 mmol/L) på dialyserbarhed samt optag og transport af jern over Caco-2 monolaget blev undersøgt fra en *in vitro* fordøjet fødevarematrix bestående af rugbrød. Fordøjelsen blev opdelt i to, med henholdsvis pepsinfordøjelse ved pH 2,0, der derefter blev inkuberet i modellen ved pH 5,5, samt direkte fordøjelse af pepsinfordøjet rugbrød med pankreatin og galdesalte ved pH 6,5 i modellen, for at simulere fordøjelse i den proximale del af henholdsvis duodenum og jejunum. Mens mælkesyre (200 mmol/L) forøgede dialyserbarheden af jern fra 12,7 ± 1,6% til 24 ± 3% (middel ± SEM, n = 4, $P < 0,05$) fra pepsinfordøjet rugbrød, var optagelsen af jern i cellerne uafhængig af mælkesyrekoncentrationen (39 ± 9 til 76 ± 11 pmol/mg protein). Derimod mindskedes dialyserbarheden af jern fra pankreatinfordøjet rugbrød fra 6,9 ± 0,2% til 2,0 ± 0,2% ($P < 0,001$), og optagelsen af jern i cellerne mindskedes fra 228 ± 30 til 8,0 ± 1,0 pmol/mg protein ($P < 0,001$), og disse to faktorer var positivt lineært korrelerede ($R^2 = 0,41$, $P < 0,01$). Grundet lav dialyserbarhed af ^{14}C-polyethylenglycol (PEG) i pankreatinfordøjet rugbrød samt kompromitteret monolag (2,4 ± 0,1%/(h × cm^2) PEG transport, n = 40), blev den transcellulære transport af jern kun estimeret for pepsinfordøjet rugbrød, og kun ved en mælkesyrekoncentration på 200 mmol/L sås en signifikant transcellulær transport af jern (180 ± 47 pmol/mg protein, $P < 0,05$). Dette resulterede i en positiv lineær korrelation mellem dialyserbarhed og total optagelse af jern fra pepsinfordøjet rugbrød ($R^2 = 0,48$, $P < 0,01$).

Tre-trins Caco-2 cellemodellen giver mulighed for detaljeret indsigt i hvert trin involveret i transporten af jern over cellemonolaget fra en fødevarematrix, og effekten af mælkesyre på absorptionen af jern fra en fytinsyrerig fødevare var hovedsagelig associeret til mængden af absorberbart jern.

Humanstudie I inkluderede 24 raske kvinder på 25 ± 4 år (middel ± SD) med lav jernstatus (serum ferritin < 40 μg/L). Testmåltiderne bestod af: (A) en mælkesyrefermenteret havrevælling, (B) en varmeinaktiveret fermenteret havrevælling, (C) en pH-justeret ikke-fermenteret havrevælling og (D) en ikke-fermenteret havrevælling tilsat organiske syrer. Måltiderne blev ekstrinsisk mærket med ^{55}Fe eller ^{59}Fe og indtaget på fire på hinanden følgende

dage, f.eks. i rækkefølgen ABBA eller BAAB efterfulgt af CDDC eller DCCD i efterfølgende periode. Absorptionen af nonhæm jern blev bestemt ud fra aktiviteten af isotoperne i blodprøver. Den fermenterede havrevælling med levende *Lp* 299v øgede absorptionen af jern signifikant ($P < 0,0001$) sammenlignet med den varmeinaktiverede og de ikke-fermenterede havrevællinger. Alle testmåltider havde samme indhold af jern, fytinsyre og polyfenoler og samme pH, mens koncentrationen af mælkesyre varierede (43-110 μmol/g). I den fermenterede havrevælling var mælkesyrekoncentrationen 19% højere end i den varmeinaktiverede fermenterede havrevælling, mens absorptionen af jern øgedes med 50%. I havrevællingen tilsat organiske syrer, var mælkesyrekoncentrationen 52% lavere end i den pasteuriserede havrevælling, mens der ikke sås nogen forskel i absorptionen af jern. Disse resultater indikerer, at foruden en effekt af de organiske syrer bidrager levende *Lp* 299v væsentligt til den øgede absorption af jern fra en mælkesyrefermenteret havrevælling.

Humanstudie II inkluderede 18 raske kvinder på 22 ± 3 år med lav jernstatus (serum ferritin < 30 μg/L). Testmåltiderne bestod af en mælkesyrefermenteret havrevælling med henholdsvis (A) levende lyofiliserede og (B) varmeinaktiverede *Lp* 299v, som blev ekstrinsisk mærket med ^{59}Fe og serveret med 2 enterocoatede kapsler (indeholdende henholdsvis $^{55}Fe(II)$ og $^{55}Fe(III)$), der var designet til at blive opløst i ileum. Måltiderne blev indtaget på to på hinanden følgende dage, f.eks. i rækkefølgen AA efterfulgt af BB i anden periode. Absorptionen af nonhæm jern blev bestemt fra helkropsretentionen af ^{59}Fe og aktiviteten af ^{55}Fe og ^{59}Fe i blodprøver. Koncentrationen af jern, mælkesyre, fytinsyre og polyfenoler samt pH var ens i de 2 måltider, og der var ingen forskel i absorptionen af jern fra måltiderne med og uden levende lyofiliserede *Lp* 299v (henholdsvis 1,4% og 1,3%). Ydermere sås ingen absorption af ^{55}Fe.

Som endelig konklusion ser den positive effekt af et mælkesyrefermenteret måltid på absorptionen af jern ud til at være forårsaget hovedsageligt af de producerede organiske syrer. Den specifik effekt af levende *Lp* 299v på absorptionen af jern i humanstudie I kunne ikke bekræftes med levende lyofiliserede *Lp* 299v i humanstudie II. Derudover sker der formodentlig

ingen absorption af jern i ileum og colon fra måltider med lav biotilgængelighed og probiotika i raske unge kvinder med lav jernstatus. Dette stemmer godt overens med resultaterne fra *in vitro* cellestudiet, der viste en positive effekt af mælkesyre på jernoptaget fra en pepsinfordøjet fødvarematrix, svarende til den proksimale del af duodenum. En modificering af fødevarer i form af mælkesyrefermentering ser derfor ud til at øge biotilgængeligheden af jern fra grøntsager og cerealier, selv om effekten i praksis er relativ lille og ikke kunne lokaliseres til en specifik effekt af levende lactobacillus.

ABBREVIATIONS

DCT1	divalent cation transporter 1
Dcytb	duodenal cytochrome b
DMT1	divalent metal transporter 1
HFE	hemochromatosis protein
IP	inositol phosphate
IRE	iron responsive element
Ireg1	iron-regulated gene 1
IRP	iron regulatory protein
Lp	*Lactobacillus plantarum*
TEER	transepithelial electrical resistance
TfR1	transferrin receptor 1
WHO	World Health Organization

NOMENCLATURE

- terms as regards iron absorption

Bioaccessibility: is the iron that is released from a food matrix during digestion and is available for absorption in the intestinal lumen, either in the form of soluble ferrous iron or iron chelated in soluble complexes.

Uptake: is the movement of a dietary compound from the intestinal lumen across the apical membrane into the epithelial cells, the first step in the process of absorption (1).

Transport: is the transfer of a dietary compound from the epithelial cells across the basolateral membrane to the circulation (1).

Absorption: is the movement of a dietary compound from the intestinal lumen across the epithelial cells of the digestive tract into the circulation (1).

Bioavailability: A generally agreed definition of bioavailability is given by Fairweather-Tait (2) as 'the proportion of the total mineral in a food, meal or diet that is used for normal body functions'. As regards iron, absorption and bioavailability can be considered equal, as there is no regulatory excretory pathway for iron. Therefore, all iron absorbed is used for normal body functions.

INTRODUCTION

Iron is one of the most abundant metals in the universe and in the earth's crust. Due to its chemical properties as a transition metal it serves as a co-factor of hemoglobin and many enzymes. It is required in all tissues of the body for basic cellular functions, and it is critically important in muscle, brain, and red blood cells.

Meanwhile, iron deficiency[1] remains one of the most common nutritional disorder in the world today, especially in the developing world, affecting an estimated 2 billion people (4), and The World Health Organization (WHO) has recently ranked it as the ninth out of the ten major global preventable risks for disease, disability, and death, and the second nutritional risk factor after underweight (5). The prevalence of iron deficiency is highest in infants, children and women of childbearing age (6-9). The high demands for iron in children is used for growth and increasing blood volume, and in women the extra needs are linked to childbirth and menstrual blood losses. In Denmark 40% of women (30-40 y) and 20% of female adolescents have low or depleted iron stores (serum ferritin < 30 μg/L) (10). The major clinical manifestation of iron deficiency is iron deficiency anemia, occurring when hemoglobin production is compromised by lack of iron to such an extent that the hemoglobin concentration falls below defined age and sex specific cut-off values[2]. Iron deficiency anemia is associated with pale skin and mucous membranes, fatigue, headache, dizziness, impaired physical capacity, and increased susceptibility to infections. In pregnant women it contributes to maternal morbidity and mortality, and increases the risk of

[1] Defined as depleted iron stores but non-anemic, *i.e.* serum ferritin concentrations < 12 μg/L and hemoglobin levels above the specific cut-off values given in footnote 2 (3).

[2] Hemoglobin levels indicative of anemia: children (½-5 y) < 110 g/L, children (6-14 y) < 120 g/L, adult females (non pregnant) < 120 g/L, adult females (pregnant) <110 g/L, adult males < 130 g/L (9).

fetal morbidity, mortality and low birth weight. Infants and children show retardation of growth and cognitive performance (9,11-13). Therefore, iron deficiency is a major health concern worldwide, and several attempts have been made in order to solve this problem.

The low iron content of several foods, mainly in the Western countries with the higher degree of industrial processing and refinement, and the limited bioavailability of iron, especially in the plant-based diets of many developing countries, are major determinants for the onset of iron deficiency. There are different strategies for reducing micronutrient malnutrition.

Iron supplements are effective in treating iron deficiency anemia and preventing iron deficiency anemia during pregnancy, which usually cannot be accomplished by dietary treatment alone. Several adverse side effects are however connected to supplementation, *e.g.* gastrointestinal distress, including diarrhea, constipation, and nausea, mainly due to the high doses of iron in the supplements given to overcome the low bioavailability, and these side effects affect compliance. In many developing countries supply and distribution problems are other factors that impair the efficiency of iron supplementation.

Food iron fortification offers a more cost-effective approach in order to provide additional iron to most parts of the population by mass fortification of staples such as wheat and maize flour, or condiments such as salt, fish sauce, or soy sauce. In addition, infants and young children can be specifically targeted through iron-fortified infant formulas and cereal-based complementary foods. However, iron is a difficult mineral to add to foods in a sufficiently bioavailable form without adverse sensory changes, and individual strategies are needed for enhancing the bioavailability and utility of iron food fortificants in specific foods (4).

One effective, but often neglected, strategy for alleviating iron deficiency is dietary modification to increase the bioavailability of the iron already present in foods (14). This can be achieved by removal of dietary components that inhibit iron absorption, such as phytate in cereals and legumes, a very strong inhibitor of iron absorption, *e.g.* by sourdough fermentation, soaking,

and germination, which lead to the activation of endogenous cereal phytases, resulting in the hydrolysis of phytate and release of iron from insoluble complexes. Or it could be achieved by increasing the intake of dietary components that enhance iron absorption from low bioavailability meals, *e.g.* by increasing the amount of enhancers such as ascorbic acid or other organic acids.

A potential way to increase the amount of organic acids is by lactic acid fermentation. Lactic acid fermented foods have led to a significant increase in iron absorption in humans (15-17), which most likely is caused by the formation of organic acids during the fermentation process. Lactic acid fermentation of vegetables and cereals is an old preservation method that is still used in developing countries, and may therefore be a natural way of increasing iron bioavailability, but also in developed countries lactic acid fermented food products has emerged in terms of probiotic foods as a part of the functional food market.

Functional foods are 'foods which are added one or a combination of components that affect functions in the body so as to have positive cellular or physiological effects' (18). These foods include probiotics, which are defined as 'live microorganisms which when administered in adequate amounts confer a health benefit on the host' (19), and prebiotics, defined as 'nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacterial species already resident in the colon, and thus attempt to improve host health' (20). The increasing use of functional foods by the public to improve their general health and prevent the incidence of chronic diseases, *e.g.* cardiovascular disease, diabetes, and cancer, has become a major area of interest within the nutritional sciences, and it has become increasingly accepted that the colonic microflora may play an important role in the maintenance of host health. Therefore, probiotic foods have gained particular interest and constitute a major part of the functional food market.

Probiotic foods include fermented foods with viable lactic acid bacteria. Several health claims have been attributed to probiotics and include benefi-

cial impact on immune status, diarrhea, other gastrointestinal conditions, cancer, and allergy (21). Lactic acid fermented foods have been shown to increase iron absorption, which leads to the hypothesis that probiotics in general may potentially increase iron bioavailability. If this hypothesis is confirmed, the exact effect and mechanism of the action remain to be investigated. The mechanism behind an increased bioavailability may be a simple pH effect, it may be the presence of organic acids in the fermented foods that act as ligands for nonheme iron, or it may be a specific activity of the probiotic bacteria, resulting in production of organic acids either in the lumen of the gut as they move along the small intestine or in the microclimate of the gut wall when colonizing the intestinal epithelium. The hypothesis and the possible mechanism need to be clarified.

AIMS

The aim of this PhD project was to evaluate the effects a specific defined probiotic lactic acid bacterium, *Lactobacillus plantarum* (*Lp*) 299v, and the fermentation products, mainly lactate, on iron bioavailability from phytate rich foods through *in vitro* and human studies to clarify the mechanism of action of these lactic acid bacteria in the human intestine.

This involved:

- Improvement and refinement of the *in vitro* Caco-2 cell model to a more suitable model, the 3-tier Caco-2 cell model, for measuring dialyzable iron, iron uptake, and transport directly from an *in vitro* digested food matrix. The new model may be used as a screening tool for determination of the effects of specific food components on the major steps in iron absorption (Paper I).
- Investigation of the effect of lactate on nonheme iron absorption from a phytate rich food matrix in the refined *in vitro* digestion/3-tier Caco-2 cell model (Paper I).
- Investigation of the effect of viable *Lp* 299v versus the fermentation products in a lactic acid fermented oat gruel on nonheme iron absorption from a phytate rich meal in a single meal crossover study with healthy young women (Paper II).
- Determination of nonheme iron absorption from a lactic acid fermented oat gruel with viable versus inactive *Lp* 299v and determination of any absorption of nonheme iron in the distal intestine in a single meal crossover study with healthy young women (Paper III).

BACKGROUND

IRON

Iron is the most abundant trace element in humans, with total body iron normally in the range 35-45 mg iron per kilogram of body weight for normal men (12). The quantity of body iron varies with body weight, hemoglobin concentration, sex, and size of the storage compartments. The largest compartment is the iron in hemoglobin, contained within the circulating erythrocytes. The size of this compartment varies considerably according to body weight, sex, and blood hemoglobin concentration. The distribution of iron in human tissue is shown in Figure 1.

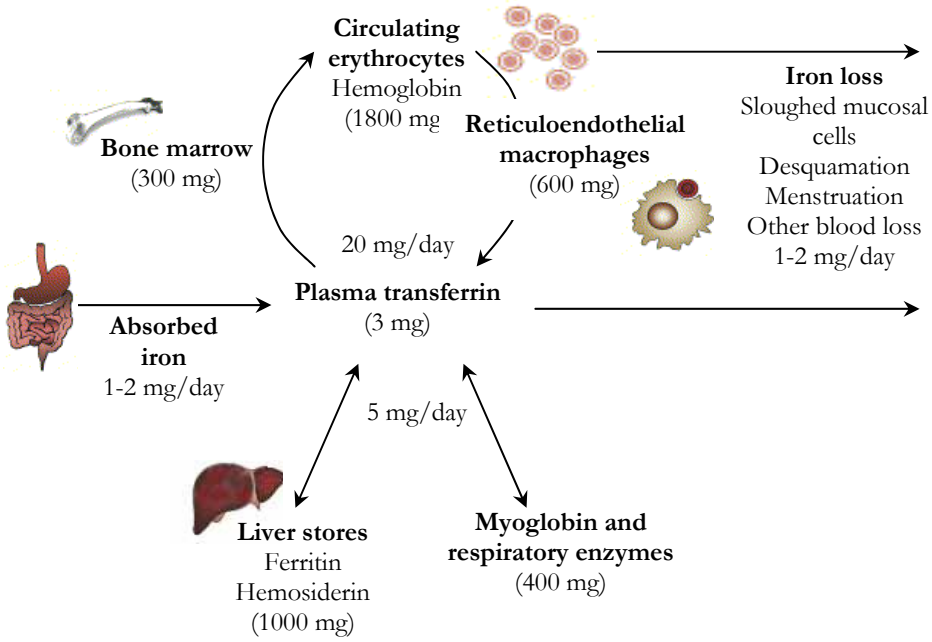

Figure 1 Systemic iron homeostasis in normal man. The major pathways of iron traffic between cells and tissues are depicted, with approximate values (significant person-to-person variation) for the iron content in the different organs given in parentheses and the daily fluxes of iron. The normal diet should contain 8-18 mg of iron daily depending on sex and age, of which only 1-2 mg is absorbed. Modified from Hentze *et al.* (22).

Iron is crucial for all living organisms because of its central role in the transport of oxygen and carbon dioxide by hemoglobin in the blood, transport and reserve supply of oxygen within myoglobin in muscle tissue, and in the functional groups of numerous enzymes of the cellular metabolism, mainly in the citric acid cycle (iron-sulphur proteins), in the respiratory chain as an electron carrier in cytochromes, in reactions of detoxification, and in the synthesis of deoxyribonucleotides (23,24). Cellular iron deficiency arrests cell growth and leads to cell death.

The biological importance of iron is mainly attributable to its chemical properties as a transition metal (oxidation states from –II to +VI), as it readily engages in one-electron oxidation-reduction reactions between its principal oxidation states, the ferric (FeIII) and ferrous (FeII) states. Whereas the solubility of Fe(II) is about 10^{-1} mol/L at pH 7, Fe(III) is much more acidic and the solubility is about 10^{-3} mol/L at pH 2 and only about 10^{-18} mol/L at pH 7 (25). This means that ferric salts are intrinsically unstable in solution unless excess acid is added and precipitates as ferric hydroxide species or is bound to ligands. Ferric iron has especially high affinity for ligands that co-ordinate through oxygen, *e.g.* phosphate, oxalate, diketonates, phenols and polyols (25). The chemical property of iron as a transition metal also explains the toxicity of an excess of free iron. In the cytoplasm, a significant fraction of iron is reduced and can participate in Fenton-type redox chemistry where ferrous iron reacts with hydrogen peroxide or lipid peroxides to generate ferric iron and the highly reactive hydroxyl radical $OH^{\cdot}$ or lipid radicals such as $LO^{\cdot}$ and $LOO^{\cdot}$ that further amplify the autocatalytic lipid peroxidative cascade. These free radicals thereby damage lipid membranes, proteins, and nucleic acids, and the pathological implications include a number of diseases, *i.e.* neurodegenerative disorders such as Parkinson and Alzheimer, atherosclerosis, and cancer, as especially seen in the inherited iron-overload disease, hereditary hemochromatosis (22,26).

Since both cellular iron overload and iron deficiency cause cell death, the systemic iron homeostasis is tightly controlled, mainly via regulation of intestinal iron absorption, but also via incorporation of iron into circulating

erythrocytes and storage proteins in bone marrow, liver, and spleen. The body's capacity to excrete iron is limited and largely unregulated (12,27).

DIETARY IRON ABSORPTION AND REGULATION

Iron absorption can be divided into three stages: Iron uptake, intraenterocyte transport and storage, and extraenterocyte transfer. These processes are controlled by both intraluminal, mucosal, and corporeal factors.

Nonheme iron is the most abundant form of iron in food, and is mostly found as ferric iron. In typical Western diets food iron occurs as 30% heme iron and 70% nonheme iron (28), and in developing countries the amount of heme iron is even less. The nonheme iron is normally in high molecular weight species and hence digestive release of iron is important for iron absorption. Furthermore, under normal intestinal conditions (*i.e.* pH 5-7 and in the presence of oxygen) ferrous iron is rapidly oxidized to ferric iron. In the luminal contents of the gut, nonheme iron is therefore poorly bioavailable, and from Western diets where the consumption of meat is high 60% of body iron is derived from absorption of heme iron (29,30).

Unlike nonheme iron, heme iron is highly bioavailable. Heme is soluble at intestinal pH and readily absorbed as an intact metalloporphyrin by enterocytes lining the absorptive villi in the duodenum and small intestine. This possibly occurs by an endosomal process after degradation of globin by the pancreatic enzymes. Once within the enterocytes the porphyrin ring is split by heme oxygenase with the release of ferrous iron (31). The released ferrous iron is then likely to enter into the pool of iron in the enterocyte and exits the enterocyte via a common pathway along with iron absorbed as nonheme iron. Whether heme iron uptake is regulated by iron status to the same extent as nonheme iron is questionable (29), and the only factor that has been reported to inhibit heme iron absorption is calcium (32). Absorption of nonheme iron therefore seems to be the more critical of the two in case of iron deficiency and iron overload. This thesis deals with the absorption of nonheme iron and will not focus further on heme iron absorption.

The term 'iron absorption' used throughout this thesis therefore only covers nonheme iron absorption.

Nonheme iron must be solubilized and chelated in the stomach to keep it in solution for absorption in the duodenum, where ferrous iron formed in the stomach otherwise readily oxidizes to ferric iron with the rise in pH. The most important role played by gastric acid secretions in nonheme iron absorption is to promote solubility of iron complexes. Various intraluminal factors affect iron absorption. Intestinal mucins (30) and enhancers in the diet, including mainly ascorbic acid, but also muscle tissue and specific amino acids (16,33,34) are important luminal accessories for iron in that they act as low-affinity ligands at neutral pH thereby maintaining it in a soluble, low molecular weight form. Ascorbic acid furthermore acts as a reductant mostly at acidic pH, reducing ferric iron to ferrous iron. Other components act as inhibitors by precipitating iron or forming macromolecules that are non-absorbable. These includes mainly phytates (myoinositol hexaphosphates (IP_6), a mineral storage molecule present in many cereals, nuts and legumes) and phenolic compounds (present in vegetables, legumes and condiments), but also phosphates (widely distributed, mainly in meats, dairy products, eggs and cereals) and certain proteins are believed to form poorly available complexes with iron (16,35,36). Calcium has long been thought of as an inhibitor of iron absorption, as shown in several single meal studies, but this effect has not been confirmed from whole-diet studies (37,38). Gastric emptying and intestinal transit time is believed to affect iron absorption, as the length of time that iron species is in contact with the duodenal surface is important for iron absorption rates. The main difficulty of addressing this factor to iron absorption is, however, to isolate transit time from other factors such as pH and type of diet (1). Iron speciation in the lumen is therefore determined by the digestion and release of iron and potential ligands and reductants from the food, together with mixing of these with ligands, acids, bases, and reductants secreted by the gastrointestinal tract (29).

Several mucosal factors affecting iron absorption have been identified. The major mechanism for regulation and transfer of nonheme iron across the

apical intestinal membrane and into the enterocytes is by the divalent metal ion transporter (DCT1 or DMT1). DMT1 is a specific ferrous ion (Fe^{2+}) transporter that is energized by the H^+ electrochemical potential gradient. Ferric ion (Fe^{3+}) is excluded. Its primary location in the gut is on the brush-border membrane of mature villous enterocytes of the proximal duodenum with a downward gradient along the small intestine (39). DMT1 is localized alongside brush-border ferrireductases, one of which is identified as duodenal cytochrome b (Dcytb). Despite progressive alkalinization of the luminal contents, the mucosal cell surface in the duodenum and proximal jejunum remains moderately acidic (40). Together with the acidic microclimate, Dcytb helps maintain significant levels of iron in its ferrous form. The microclimate also provides the proton electrochemical potential gradient as the motive force for iron transport across the apical membrane within DMT1 (39). As for DMT1 the activity of Dcytb is expressed mainly in the duodenum with a downward gradient along the small intestine, similar to the profile of iron absorption along the gut. The expression of DMT1 and Dcytb is closely correlated and they are tightly regulated by both local enterocyte levels, leading to rapid downregulation after high oral doses of iron, the so-called 'mucosal block' phenomenon (41), and body iron status, leading to upregulation under conditions of iron deprivation (29). Nevertheless, only the gene encoding DMT1 contains a recognizable iron responsive element (IRE) that confers iron dependent regulation by interactions with iron regulatory proteins (IRP1 and IRP2). DMT1 may therefore represent a key mediator of intestinal nonheme iron uptake (29,42). A further role of DMT1 as a handoff molecule for iron both in the lumen of the intestine and on the basolateral side to apotransferrin, due to its localization elsewhere in or near the enterocytes, remains to be explored (39). The pathway for intestinal nonheme iron absorption is shown in Figure 2.

Little is known regarding the intracellular transport of iron within the absorptive enterocyte. Iron is either stored as ferric iron by ferritin, a ubiquitous and highly conserved protein, or transferred across the basolateral membrane to reach the plasma. The excess protons co-transported with Fe^{2+} within DMT1 may contribute to a localized intracellular acidification,

maintaining the chemical state of Fe^{2+} before it is handed of to an intracellular chaperone (39). Iron that remains bound to ferritin as the enterocyte completes its limited life cycle (2-4 d) will be sloughed with the senescent cell and will leave the body through the gut. This process represents an important mechanism of iron loss (12). The basolateral transfer of iron requires the transfer protein ferroportin/iron-regulated gene 1 (Ireg1) and hephaestin, a multicopper ferroxidase, presumably situated at the basolateral surface of the cell.

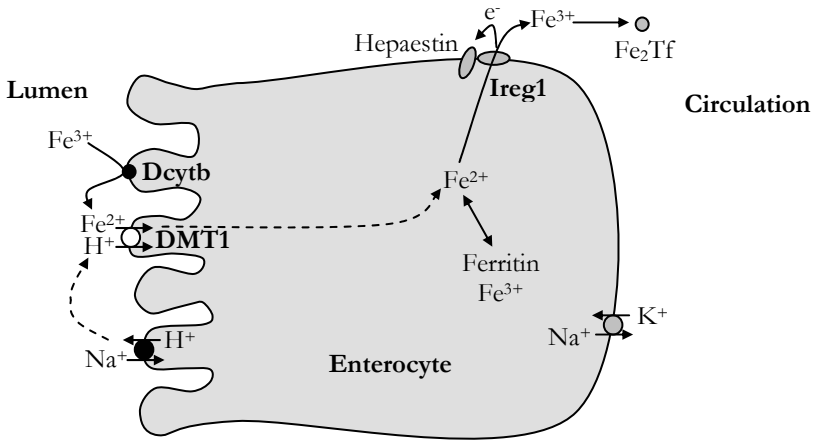

Figure 2 Pathway for intestinal nonheme iron absorption. Fe^{3+} is reduced to Fe^{2+} by ascorbic acid and apical membrane ferrireductases, including duodenal cytochrome b (Dcytb). The apical microclimate at the brush border provides an H^+ electrochemical potential gradient to drive transport of Fe^{2+} via the divalent metal ion transporter DMT1 into the enterocyte. Basolateral transport of Fe^{2+} to the circulation is mediated by Ireg1/ferroportin1 in association with hephaestin. Fe_2Tf, diferric transferrin. Modified from Mackenzie & Garrick (39).

Questions remain unanswered regarding the mechanisms of iron transport by Ireg1, but the protein is a member of the solute linked carriers protein family suggesting transport of iron within Ireg1 either by a facilitated diffusion mechanism or by the oxidation of ferrous iron to ferric iron by either hephaestin or ceruloplasmin. Hephaestin is highly expressed along the length of the gut whereas Ireg1 is expressed predominantly in the duodenum like DMT1 and Dcytb, but also in the colon is there a significant expression of Ireg1 (29). However, in contrast to DMT1, the expression of

Ireg1 and Hephaestin is regulated by systemic rather than local signals of iron status. The basolateral transport step therefore appears to be the primary site at which the enterocytes responds to systemic stimuli and alterations in body iron requirements (43). From the basolateral membrane iron is transferred in the blood as diferric transferrin. Transferrin is a plasma glycoprotein, which binds two ferric iron atoms with high affinity, and provides most of the iron for the physiological needs of iron-requiring cells. Iron is transported with transferrin to the muscle tissue for utilization, to the bone marrow for erythropoiesis or stored within ferritin or hemosiderin in hepatocytes or tissue macrophages (12).

As described above the maximal rate of iron absorption occurs in the duodenum and then decreases from the proximal gut to the ileum, along with the expression both of DMT1, Dcytb, and Ireg1. However, the lower portion of the intestine may also play an important role in the absorption of iron, especially during iron deficiency (44-47). Iron absorption has been shown to increase from the ileum to the caecum and proximal colon in rats, and the ratio between absorption rates in the duodenum and proximal colon was about 2 on a surface basis. With the larger surface area of the colon and the longer transit time and therefore longer period of time for exchange than in the duodenum, the colon may add a significant contribution to the absorption of iron (44). Results from a dog study with infusion of radiolabeled iron solutions directly in the duodenum or cecum of iron-replete and iron-deficient dogs, respectively, suggest that the colon could be a significant site for iron absorption in iron deficiency but not in iron adequacy (48). Consistent with these findings recent work in mice and rats have shown significant expression both Dcytb, DMT1 and Ireg1 in the large intestine, which increased during iron deficiency (47,49). Only one study reports on the measurement of iron absorption directly from the colon in humans. Ohkawara *et al.* (50) presented evidence that human subjects are capable of absorbing soluble ferrous iron but not ferric iron directly from the large intestine by infusion of radiolabeled ferric and ferrous chloride solutions directly into the colons of non-anemic human subjects through a sigmoidoscope. The above-mentioned studies indicate that although iron

absorption from the colon is less efficient than from the proximal small intestine, there seems to be a significant absorption of iron from the colon, especially in the condition of iron deficiency. Although very little is known about the mechanisms of iron absorption in the colon, the colonic mucosa seems to be a highly permeable membrane that absorbs iron via both active and passive diffusion (45). The luminal pH, the caecal surface area, and the concentration of organic acids in the colon are closely associated with the quantities of fermentable carbohydrates, as well as mineral concentrations (51), thus, microbial fermentation may play a major role in iron absorption from the colon.

Dietary iron absorption is regulated, in part, in response to corporeal signals generated at the body's main sites of storage (the liver) and utilisation (the bone marrow) – these are often called the 'stores regulator' and the 'erythroid regulator', respectively. One of the central unresolved questions in iron metabolism is how the duodenal mucosa is able to sense this level of body iron stores or changes in demands for iron, and various hypotheses for the mechanisms have been proposed.

It is suggested that the iron requirements of the body is initially sensed by the duodenal crypt cells by delivery of transferrin-bound iron from the plasma to the intestinal cell by the transferrin receptor (TfR1) on the baso-lateral surface in the presence of HFE, the hemochromatosis protein, in complex with β_2-microglobulin. The transferrin-TfR1 complex is then internalized via receptor-mediated endocytosis resulting in the formation of endosomes. Acidification of the endosome releases ferric iron from transferrin and reduces it to ferrous iron and the iron is then most likely transferred from the endosome to the cytosol of the crypt cells via DMT1. TfR1 expression is controlled by intracellular levels of iron via IRP1 and IRP2 (52). The crypt cells are then programmed by that information as they mature into absorptive enterocytes lining the absorptive villi (53) thereby acting as a stores regulator.

Another recently identified key stores regulator is hepcidin, an antimicrobial peptide secreted into the plasma from the liver, and excreted through the

kidneys. Hepcidin has been proposed to act both as a 'store regulator', an 'erythroid regulator', a 'hypoxia regulator', and an 'inflammatory regulator' (22). It is a powerful negative regulator of iron absorption that inversely correlates with DMT1 and Dcytb expression. It appears to communicate body iron status and demand for erythropoiesis to the intestine, and in turn, modulates intestinal iron absorption (54,55). The exact interactions still remain to be determined but suggestions have been made that it might interact with the HFE-β_2-microglobulin complex and increase iron uptake in duodenal crypt cells from the basolateral compartment, thereby programming them as they differentiate into mature enterocytes (29) or it might bind directly to Ireg1 or an associated transport molecule on the mature enterocytes (22).

The finding of hepcidin has shed some light on the regulatory system involved in iron metabolism, but many aspects of iron homeostasis still remains to be clarified.

PRO- AND PREBIOTICS IN IRON ABSORPTION

Probiotics

Due to the attributed health benefits of probiotics a growing commercial interest in the probiotic food concept, which constitutes a sizeable part of the functional food market, has been observed in recent years. However, major concerns regarding the quality, labeling and verification of health claims attributed to these products still exist. An upsurge in the research into probiotics has resulted in significant advances in our understanding and ability to characterize specific probiotic organisms, as well as attempts to verify their attributed health benefits. This has resulted in scientific evidence that specific strains of probiotic microorganisms confer benefits to the health of the host and are safe for human use, but much work still needs to be done to ensure that the credibility of the health claims lasts and internationally uniform regulatory procedures for probiotic foods are required (21).

The main microorganisms used as probiotics are species of the genera *Lactobacillus* and *Bifidobacterium*, which are Gram-positive bacteria naturally oc-

curring in the human gut (56). Furthermore the genera *Escherichia*, *Enterococcus*, and *Saccharomyces* have been used. The latter do however give rise to concerns regarding safe use (21). Probiotics must be able to exert their benefits on the host through growth and activity in the human body, *i.e.* they must be capable of surviving passage through the gut by the ability to resist gastric juices and bile and they must be able to proliferate and colonize the digestive tract. Furthermore they must be safe and effective. It is however the specificity of action and not the origin of the microorganism that is important (19).

The beneficial health effects attributed to probiotics include: a) the ability of the probiotic bacteria to modulate the immune system by promoting the endogenous host defense system, including the humoral, cellular and non-specific immune response, b) treatment of diarrhea by reducing the incidence or duration of certain diarrheal illnesses, the most substantiated health claim today, c) prevention and treatment of other gastrointestinal conditions such as *Helicobacter pylori* infection and inflammatory bowel diseases, *i.e.* ulcerative colitis and Crohn's disease, d) protection against carcinogenic activity, e) reduction in the risk of allergy as probiotics may reverse increased intestinal permeability, promote gut barrier function and enhance the gut-specific IgA response and production of cytokines, and f) reduction in the risk of genitourinary tract infections (19).

The incorporation of probiotic bacteria in foods has been focused mainly on dairy products, even though many traditional lactic acid fermented foods besides milk-based ones are available. Examples of traditional, non-milk based foods containing high concentrations of lactobacilli are brined olives, salted gherkins, and sauerkraut, *i.e.* lactic acid fermented plant materials, where the fermentation process is carried out by a spontaneous mixed flora. The bacterial composition of such lactic acid fermented foods normally constitutes different lactobacillus strains, especially *Lp*, as they are more acid resistant than other lactic acid bacteria, being able to grow well at pH values around 4-5, and therefore responsible for the final stages of most lactic acid fermentations. For one reason or another, these lactic acid fermented foods are rarely considered as carriers for probiotic bacteria (57). One of the few

nondairy probiotic food products on the market is a lactic acid fermented fruit drink (ProViva, Skåne Dairies, Malmö, Sweden), which is based on oat gruel fermented with the patented probiotic strain *Lp* 299v (58), with all licences owned by Probi AB (Lund, Sweden). The strain has been isolated from healthy human intestinal mucosa (59), and has been shown to survive passage through the human gastrointestinal tract and to colonize the intestine for a shorter period of time (60). The following probiotic effects have been reported for *Lp* 299v in clinical trials: a) decreased translocation of intestinal bacteria, b) competition with *Enterobacteriaceae* in the intestinal mucosa, c) improved liver status during liver injury, d) improved immunologic status of the mucosa, e) decreased inflammation in the mucosa, and f) modulation of the responsiveness to antigens presented via the gut (57).

Several lactic acid fermented foods have shown to increase iron absorption in humans. Derman *et al.* (15) showed a significant increase in iron absorption from maize and sorghum beer both compared to the constituents used to prepare the beer, but also from a gruel of similar pH containing lactic acid, in single meal human studies. Two other single meal human studies have shown that compared to other vegetables and whole meals, sauerkraut had the highest increase in iron absorption (16,17). Brune *et al.* (61) showed that sourdough fermentation increased iron absorption. As the lactic acid bacteria have been inactivated by heat treatment before ingestion of these tested meals, the enhancing effect of these lactic acid fermented foods on iron absorption is believed to be due mainly to the small organic acids that are produced during the lactic acid fermentation, with lactate as the major end product. The organic acids contribute to several characteristics of the fermented foods that are positive for iron absorption: a) the lowering of pH, which activates endogenous phytases present, thereby leading to breakdown of phytate and release of iron from insoluble iron-phytate complexes (36,62), which is probably the most important factor, b) the buffer capacity of lactic acid in the pH-range 3-5, which may hinder or delay the formation of less soluble iron compounds such as ferric hydroxide with reduced bioavailability (17), c) the possible formation of soluble iron-organic acid complexes at intestinal pH (15,17), and d) the delayed gastric emptying rate

(63), which leads to an increase in the length of time that iron is in contact with the duodenal surface (1,64).

Whether lactate is able to act as a specific enhancer of iron absorption by formation of iron-lactate complexes, that are soluble and highly bioavailable at physiological pH, remains questionable (65). A specific effect of lactic acid on iron absorption has been shown in the study of Derman *et al.* (15), with an increase in iron absorption from a low pH gruel (3.5) added lactic acid compared to a low pH gruel with hydrochloric acid, but iron absorption from a fermented beer was still significantly better than from the gruel of similar pH with added lactic acid. However, no affect on iron absorption was seen when lactic acid was added to rice (66). *In vitro* Caco-2 cell studies of the effect of lactic acid on iron uptake and transport has not been able to fully confirm an enhancing effect of lactic acid on iron absorption, as the results revealed a dose-dependent inhibition in the uptake of both ferrous and ferric iron, whereas the transport of ferric iron increased (67,68).

The increased iron absorption from certain lactic acid fermented foods indicate that, in addition to the health benefits attributed to probiotics due to colonization, growth and activity in the gut, probiotic lactobacilli may potentially increase iron bioavailability either through fermentation of the food products before ingestion, thereby supplying low pH foods with high concentrations of organic acids, or by activity in the small intestine either as they move along or after colonization.

Prebiotics

Nondigestible oligosaccharides in general, and fructooligosaccharides and oligofructose (inulin) in particular, are prebiotics, in that they modify the composition of the colonic microflora in such a way that a few of the potentially health-promoting bacteria (especially lactobacilli and bifidobacteria) become predominant in numbers (14). Prebiotics and their products of fermentation by the intestinal microflora have been suggested to affect mineral bioavailability and lipid metabolism, and reduce the risk of developing precancerous colonic lesions (69). Although it is widely accepted that prebiotics improve calcium absorption (20), the effect on iron absorption and

the mechanisms involved are poorly understood (14,20). Rat and pig studies have provided some evidence supporting the hypothesis that prebiotics enhance iron absorption, as iron absorption has been shown to increase with the ingestion of both fructooligosaccharides and inulin (70-74) and resistant starch (75), especially in iron-deficient animals. However, this effect has not been confirmed in humans in the limited number of human studies published. Ellegård *et al.* (76) showed that when feeding fructooligosaccharides or inulin to ileostomy subjects, 88% and 89%, respectively, were recovered in the ileostomy effluents, confirming that fructooligosaccharides are resistant to digestion in the human stomach and small intestine. The fructooligosaccharides did though not affect the apparent absorption of iron nor calcium. The same tendency was observed by Van den Heuvel *et al.* (77), where the intake of inulin, fructooligosaccharides or galactooligosaccharides did not interfere with calcium and iron absorption. In another study, addition of inulin to a control diet did not increase the apparent iron absorption, whereas calcium absorption increased significantly (78). In a recent study involving 634 Indian children, fortification of a milk formulation with *Bifidobacterium lactis* and galactooligosaccharides resulted in a 35% reduction in the proportion of iron-deficient children (79). Whether this improvement was due to an enhanced iron absorption or due to reduction of bloody diarrhea, as a result of healthier intestinal microflora, is however unclear. As discussed by Yeung *et al.* (14) the lack of effect of prebiotics on iron absorption in the study by Van den Heuvel *et al.* (77) could be due to the high intake of ascorbic acid (300 mg/day), which may overwhelm the effect of the prebiotics. Furthermore, in the studies by Van den Heuvel *et al.* (77) and Coudray *et al.* (78) the subjects were young healthy men with adequate iron status. If the control diets in the studies provided sufficient bioavailable iron to meet the subjects' requirements, an increase in bioavailability may not produce a detectable increase in absorption (14).

Apart from the general benefits associated with their consumption, several plausible ways for pro- and prebiotics to enhance iron absorption have been hypothesized: a) organic acids produced by fermentation in the colon may facilitate iron absorption by lowering luminal pH and improving iron solu-

bility, b) short-chain fatty acids produced by fermentation in the colon may stimulate the proliferation of epithelial cells, thereby increasing the absorptive surface area in the colon, and c) prebiotics or fermentation products may stimulate the expression of iron regulatory genes, thereby increasing iron absorption (14,18). Many questions regarding the possible effects of pro- and prebiotics on iron absorption do though remain unanswered, and a better understanding of their potential effects and mechanisms of actions involved need to be clarified (14).

METHODS AND METHODOLOGICAL CONSIDERATIONS

While the concept of iron bioavailability is fairly simple, the problems arise in its determination, as it involves various stages, *i.e.* availability, uptake, transfer across the basolateral membrane, utilization, and storage, each of which is affected by either dietary or physiological factors. A number of *in vitro* and *in vivo* methods have been used to assess nonheme iron bioavailability. Long term human intervention trials are the 'golden standard' in human nutrition, and should normally constitute the final stage when investigating the influence of dietary factors on iron bioavailability. However, these studies are very expensive and time-consuming. Normally, single-meal studies are the first step when going into the field of human trials. The use of radioisotopes or stable isotopes is the most reliable method for determining iron bioavailability in humans over a short period. However, besides the ethical considerations, particularly with the use of radioisotopes, they are also expensive and time consuming (2).

Many attempts have been made to set up *in vitro* models to assess the bioavailability of iron from different foods and diets. These models mainly include simulated digestion followed by determination of solubility, dialyzability and/or cell uptake and transport, and can be used as a first indicator of the influence of specific food components, foods, or diets on iron bioavailability in that they give a measure of the first stages in bioavailability, *i.e.* availability and uptake. Nevertheless, the final stage of iron bioavailability, utilization, is not determined in these models and requires the use of animal models, of which rat models most often are used for iron absorption studies. With differences in mineral requirements, metabolism, digestion, physiology, and sensitivity to dietary factors, the results are though not quantitatively comparable to humans (80).

Another approach to estimate iron bioavailability is calculations using algorithms to predict the bioavailability of iron from mixed diets (81), which gives semi-quantitative information such as classification of diets into high, moderate, or low bioavailability. These algorithms are based on limited data from human studies on the effects of enhancers and inhibitors on iron absorption, and their accuracy depends on the quality of the data used to develop them and the accuracy and availability of food composition data for iron, enhancers, and inhibitors (82).

THE *IN VITRO* DIGESTION/CACO-2 CELL MODEL

The Caco-2 cell line

The Caco-2 cell line is a cell line derived from a human colonic carcinoma that spontaneously exhibits signs of structural and functional differentiation and polarization *in vitro* when grown under standard conditions. This is characterized by typical brush border microvilli extending perpendicularly to the surface from the upper side of the monolayer, formation of domes that is typical of transporting epithelial monolayers, and high levels of activity of the brush border associated enzymes such as alkaline phosphatase, sucrase-isomaltase, lactase, and amino- and dipeptidylpeptidases and also expression of growth factor receptors (83-85). Furthermore, when grown on collagen-coated polycarbonate membranes, the Caco-2 cells exhibit well defined tight junctions and desmosomes and possess similarities with the human small intestinal epithelium in both uptake and barrier properties (86). With proliferation, confluency, and differentiation, the growth of Caco-2 cells *in vitro* is very similar to the renewal of the small intestinal epithelium, characterized by proliferation within the crypt and differentiation during migration to the villus tip *in vivo*, which makes the Caco-2 cell line a valuable transport model system for the human small intestinal epithelium.

As for most *in vitro* models there are disadvantages of using the Caco-2 cells as a model of the human small intestinal epithelium. Firstly, the transformed nature of the Caco-2 cells originating from a human colon carcinoma. It remains questionable to what extent normal metabolic processes relative to

man are maintained in these cells (80). Furthermore, only a minor fraction of the small intestine will be simulated, different cells such as goblet cells, paneth cells and crypt cells present in the gut are less organized and therefore leakier, the transepithelial electrical resistance (TEER) is higher than in the human small intestine and resembles that of human colon (85), there is no regulatory control by neuroendocrine cells and through the blood (87), no mucin layer is present (80), some characteristics of lipid metabolism differs depending on growth conditions (85), and finally, even though the Caco-2 cells express many of the transport proteins that are used to facilitate uptake of otherwise poorly absorbed nutritional compounds, they have lower carrier expression resulting in lower transport rates, so that a scaling factor may be required (80,85).

Iron uptake and transport in Caco-2 cells

Because of the human origin, the highly polarized monolayer, the many similarities with small intestinal cells, and the vectorial transport, Alvarez-Hernandez *et al.* (88) introduced the use of Caco-2 cells for studying iron uptake and transport, and the cell line has since then been shown to mimic the effects of *in vivo* food iron absorption in several ways. Iron uptake in these cells is affected by the iron status of the cells (88,89) and the valency of iron, with a preference for ferrous iron (88,90). Furthermore, IP_{3-6} inhibit the uptake and transport of iron by Caco-2 cells (91), and ascorbic acid offsets this inhibitory effect (92).

As for mammalian enterocytes the Caco-2 cells have an active IRE/IRP system that is regulated by the amount of intracellular iron (93,94). This system is responsible for the regulation of expression of ferritin and TfR, and with the polarized distribution of TfRs, dependent on the differentiation process (95), the Caco-2 cells are able to acquire transferrin-bound iron from the basolateral compartment by an endocytic pathway (96), thereby regulating cellular levels of ferritin and apical iron uptake (97-99). HFE needed for the internalization of TfR is also present in the Caco-2 cells and regulated by the iron status of the cells (100). The major apical and basolateral iron transport molecules, DMT1 and Ireg1, have also been shown to

be abundantly expressed in these cells and regulated by iron treatment (100-105) and DMT1 also by the regulatory peptide, hepcidin (106). Furthermore, the Caco-2 cells are able to reduce ferric iron by the plasma membrane ferrireductase Dcytb (104,107).

In summary, the various iron-related proteins are present in the Caco-2 cells and show the same overall trend in regulation by the most important intraluminal, mucosal, and corporeal factors as in humans.

Combined *in vitro* digestion and absorption in Caco-2 cells

Early studies on iron bioavailability dealt with the availability stage by *in vitro* determination of ionic iron in aqueous food extracts. Food iron solubility studies have since then developed to a determination of total soluble iron from foods, *i.e.* either ionic iron or soluble complexed iron, that have undertaken a simulated gastrointestinal digestion with pepsin at pH 2 followed by neutralization and digestion with pancreatin and bile salts (80). Coupling of this simulated digestion with dialysis as introduced by Miller *et al.* (108) is a frequently used method that highly correlates with human iron bioavailability trials with similar foods (109). However, the gastrointestinal physiology is very complex, and iron solubility under simulated intestinal conditions may not accurately predict iron availability in all cases. Effects of transit time, enzyme concentrations, fluctuations in pH, sites of absorption, motility, and diffusion barriers may for example not be accounted for sufficiently *in vitro* (110).

The combined *in vitro* digestion and absorption in Caco-2 cells was introduced by Gangloff *et al.* (111), Glahn *et al.* (112), and Garcia *et al.* (113) ten years ago. Whereas Garcia *et al.* (113) used centrifugation of the pepsin digested food matrix before adding the supernatant onto the Caco-2 cell monolayer grown on microporous membranes, allowing assessment of both uptake and transport across the membrane, Gangloff *et al.* (111) used the *in vitro* digestion and dialysis method (108), followed by incubation of the dialyzed food samples on the Caco-2 cell monolayers grown on cell culture plastic. Glahn *et al.* (112) on the other hand carried out the availability and uptake experiments simultaneously, by conducting the *in vitro* pancreatin-

bile digestion directly in the Caco-2 cell model with the insertion of a dialysis membrane on top of the cell monolayer grown on cell culture plastic (Figure 3). This method has a number of benefits, in that it prevents damage to the cells from the digestive enzymes, recreates a dynamic process close to that found in the small intestine above the Caco-2 monolayer, and the dialysis step, which models exchange at the unstirred layer, involves small volumes compared to those used historically in many dialyzability experiments (108). A disadvantage of the technique is that it cannot predict basolateral exit from the cell, which is important in understanding how much iron reaches the blood stream. Also, the fact that the efflux pathway is blocked may alter the uptake ratio of iron into the cell.

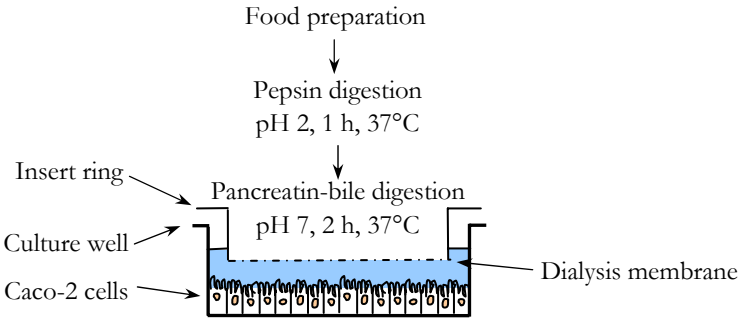

Figure 3 Diagram of the *in vitro* digestion/Caco-2 cell model developed by Glahn *et al.* (112).

This combination of *in vitro* digestion and uptake studies in Caco-2 cells has been used extensively the last decade for determination of the accuracy of the model in predicting the effects of different dietary components on iron bioavailability in humans, such as ascorbic acid, meat proteins, phytate, polyphenols, and calcium (82,114-119), and as a screening tool for selecting plant varieties with high iron bioavailability (120,121). The model has demonstrated qualitative similarities to human trials, and recently ascorbic acid and tannic acid have also shown to respond quantitatively to human trials when comparing absorption ratios (82). This Caco-2 cell model is therefore the model that closest approaches an estimation of iron bioavailability *in*

vitro since it measures both iron dialyzability and uptake in the human intestinal Caco-2 cell line, and takes into account some of the physiological factors that influence iron absorption by introducing the dialysis membrane, and the validity of extrapolating from animals to humans are avoided. Furthermore, the costs of carrying out these *in vitro* studies are less than studies in animals and in humans in particular, and also less time consuming, thus with these *in vitro* cell studies it is possible to approach the main variables of interest before carrying out *in vivo* studies, which is also more ethical in that the number of *in vivo* studies often is reduced.

The incorporation of the dialysis membrane in the model is of great importance for protecting the cells from the digestive enzymes, why the model developed by Glahn *et al.* (112) is preferable to the model developed by Garcia *et al.* (113). Even though the model has shown to correlate well with human trials regarding iron bioavailability, the lack of ability to measure transport is a disadvantage of the model when it comes to mechanistic studies of dietary components affecting iron absorption and explanations for effects observed *in vivo*. Furthermore, the value and importance of corporeal factors such as hepcidin and transferrin, which have shown to be major determinants of dietary iron absorption (29), cannot be investigated in this model. Therefore, the aim of this *in vitro* study was to improve and refine the Caco-2 cell model developed by Glahn *et al.* (112), primarily by adding another tier in the system, the microporous membrane, making it a 3-tier system with the ability to measure both dialyzability, uptake, and transport of iron from *in vitro* digested food matrices simultaneously (Figure 4). Secondly, as iron is claimed to be absorbed primarily in the duodenum and most proximal part of jejunum, the model was set up to measure iron absorption from a peptic digest (pH 5.5) and a pancreatic digest (pH 6.5) *in vitro*, corresponding to the digestion conditions in the lumen of the proximal duodenum and jejunum, respectively (122). The suitability of using this model was assessed by investing the effect of lactate (0-200 mmol/L) on nonheme iron dialyzability, uptake and transport from *in vitro* digested rye bread, and the exact experimental procedure is described in Paper I.

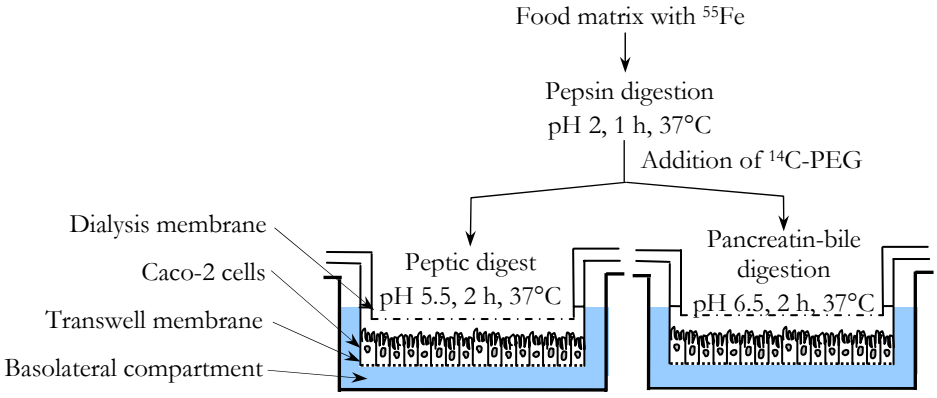

Figure 4 Diagram of the modified *in vitro* digestion/3-tier Caco-2 cell model.

An additional kinetic study in the 3-tier Caco-2 cell model was carried out as a separate experiment to study the permeability of mannitol across the monolayer during the incubation period (data not included in paper I). The TEER of the Caco-2 cell monolayers (passage 26) was measured during the cell culture period and prior to the experiment (day 21) at room temperature, using Endohm cups and an EVOHM epithelial voltohmmeter (World Precision Instruments, Sarasota, FL). ^{14}C-mannitol was used as a non-absorbable marker and was added directly to the apical compartment, and the permeability of ^{14}C-mannitol across the Caco-2 cell layer was determined by taking out aliquots (100 μL) from the basolateral compartment 10 min after beginning of the incubation period and then in 30 min intervals over the 120 min incubation period. In the experiment four different concentrations of lactate (0-100 mmol/L, n = 6 for each) were investigated in both peptic and pancreatic digests, and the experiment was, in any other respect, carried out similarly to the study described in Paper I.

HUMAN STUDIES USING RADIOISOTOPES

A number of methods have been employed to assess iron bioavailability in humans, such as a) the chemical balance technique, b) the rate of repletion following depletion, c) plasma appearance, d) radioisotope and stable iso-

tope techniques, and e) change in iron status. The chemical balance technique and measurement of change in iron status measures iron bioavailability only from whole diets, the rate of repletion technique is difficult to organize, but could be useful in studying the efficiency of different food sources in treating iron deficiency anemia, plasma appearance measurements are mostly used for highly concentrated sources of iron, such as iron supplements, to answer pharmacological questions, and a change in iron status requires a long-term intervention. On the contrary, the radioisotope and stable isotope techniques can measure iron absorption from whole diets or single meals. The use of radioisotopes has made considerable progress in the field of iron bioavailability, and have a number of advantages compared to the other methods (2). They are true tracers and do therefore not change the chemical balance of the food, the detection of radioisotopes is relatively simple and measurements can be made with high precision, dual radioisotope techniques can be used, and it is possible to conduct *in vivo* measurements (123). These advantages have to be balanced against the potential hazards of radiation, and even though there is no consensus with regard to the ethical considerations involved in the use of radioisotopes for nutritional research, it is generally agreed that radioisotopes should not be administered to pregnant or lactating women, or to infants and children (2).

The use of extrinsic radiolabels is a valid technique for the measurement of food iron bioavailability (124) and has made it possible to study individual foods without biosynthetical labeling (2). The method is based on the assumption of an almost complete isotopic exchange between iron compounds in the food and the added inorganic radioiron tracer, which has been documented for a number of foodstuffs (125) and is therefore expected to give a true measure of the iron absorption from the meal. The 'true' retention of iron in the body can be measured *in vivo* by whole-body counting without sampling. This method is therefore considered as the 'golden standard' for isotope measurements *in vivo* (123). The whole-body counter does however only measure the retention of γ-emitters, such as ^{59}Fe and not the low energy of the electron capture decay of ^{55}Fe. This measure of retention applies well to iron, as the excretion of absorbed iron in the

experimental period between intake and measurement, where the non-absorbed fraction of the labeled meal is excreted, is negligible (2,123).

By using both radioiron isotopes, ^{55}Fe and ^{59}Fe, which allows dual labeling of 2 meals, also known as the dual label extrinsic tag method (125), iron absorption into the blood can be measured of from 2 meals simultaneously by analyzing the activity of ^{55}Fe and ^{59}Fe in a blood sample (126) drawn approximately 2 weeks after administration of the test meals. The total absorption is normally estimated based on the anticipation that 80% of the iron is incorporated into hemoglobin ≥ 8 d after ingestion of the isotope (127) , and the blood volumes of the subjects has to be estimated from body weight and height (128).

A unique technique to measure iron absorption from several meals is by combining the measurement of ^{55}Fe and ^{59}Fe activities in blood samples with whole-body counting of total ^{59}Fe retention. In this way the ^{55}Fe retention in the whole body can be estimated, when it is anticipated that the incorporation of ^{55}Fe and ^{59}Fe into hemoglobin is equal (125).

A major problem with iron absorption studies is the marked variability in absorption in different subjects due to differences in iron status. This problem can be overcome by the use of crossover studies, such that each subject serves as its own control, whereby absorption ratios for different meals can be compared within a study. When comparing with other studies the variation in absorptive capacity can be taken into account for by determining absorption from a standard dose of inorganic radioiron given under standardized conditions. The ratio of mean absorption from a meal and from the reference dose is a measure of the relative bioavailability of nonheme iron in the meal. An alternative to reference dose absorption is the use of serum ferritin, which is a good indicator of iron status and correlates well with iron absorption. Another problem in iron absorption studies is the day-to-day variation in absorption within the same subject. This can be overcome by serving the tagged meal twice on two consecutive days (129).

Table 1 Study design of the single meal human studies I and II.

		Period I					Period II					
Human study I	Day	1	2	3	4	22	23	24	25	26		43
(Paper II)	Test meal*	A	B	B	A	🩸	C	D	D	C		🩸
(n = 24)		^{55}Fe	^{59}Fe	^{59}Fe	^{55}Fe		^{59}Fe	^{55}Fe	^{55}Fe	^{59}Fe		
Human study II	Day	1	2			17	20	21	22		38	41
(Paper III)	Test meal* 🧍	A	A			🧍	🩸	B	B		🧍	🩸
(n = 18)		^{59}Fe	^{59}Fe					^{59}Fe	^{59}Fe			
	Enterocoated capsules†	^{55}Fe	^{55}Fe					^{55}Fe	^{55}Fe			

* Test meals: (A) fermented oat gruel with viable *Lp* 299v, (B) fermented oat gruel with inactive *Lp* 299v, (C) non-fermented oat gruel, pH adjusted, and (D) non-fermented oat gruel with organic acids. All serving orders of the meals were used and the subjects were randomly assigned to each of these groups.
† Two capsules containing ^{55}Fe(II) and ^{55}Fe(III), respectively, were served with each meal.
(🧍) Whole-body counting, (🩸) blood sampling.

Both human studies described in this thesis were single meal, double-blinded, randomized, crossover studies in young healthy women of child-bearing age (Table 1). The crossover study design was chosen to obtain a strong design with relatively few subjects. In human study I the effect of viable *Lp* 299v versus the fermentation products in a lactic acid fermented oat gruel on nonheme iron absorption from a phytate rich meal was investigated (Paper II). A fermented oat gruel with viable *Lp* 299v (A) was tested against two test meals, which were prepared to have the same concentration of organic acids, *i.e.* a heat-inactivated fermented oat gruel (B), which in addition to the organic acids also contained other components produced during the fermentation procedure, such as *e.g.* peptides and amino acids, and a non-fermented oat gruel with added lactic and acetic acid (C) in concentrations estimated to be obtained in the fermented oat gruel. Finally, a non-fermented oat gruel (D) pH adjusted to the same pH as the fermented oat gruel was included as a control for an effect of pH. The four oat gruels were all prepared from the same batch before the first period and the production is outlined in the flow diagram in Figure 5. With 4 test meals in human study I the dual label extrinsic tag method was used with determina-

tion of iron absorption from activities of ^{55}Fe and ^{59}Fe in blood samples (Table 1). In human study II the effect of viable *Lp* 299v in the fermented oat gruel on nonheme iron absorption was investigated, with measurement of any iron absorbed in the ileum and colon (Paper III) and only two test meals were relevant: (A) a lactic acid fermented oat gruel with viable *Lp* 299v versus (B) a lactic acid fermented oat gruel with inactive *Lp* 299v. The production of the fermented oat gruel with the viable *Lp* 299v was modified from human study I (Figure 5) by adding viable lyophilized *Lp* 299v to the heat-inactivated lactic acid fermented oat gruel (B) one day before ingestion instead of using the active fermented oat gruel during the whole intervention, as the activity of *Lp* 299v during storage (4°C) between the 2 periods was too high why the concentration of organic acids increased. By using the simple crossover study with one test meal in each of 2 periods only one radioiron isotope (^{59}Fe) was necessary for extrinsic labeling of the test meals. The second radioiron isotope (^{55}Fe) was used for determination of iron absorption in the ileum and the colon by including 2 enterocoated capsules designed to disintegrate in the ileum. The 2 capsules were labeled with $^{55}Fe(II)$ and $^{55}Fe(III)$, respectively. For practical reasons there was no randomization of ^{55}Fe and ^{59}Fe between the meals and the enterocoated capsules (Table 1). The exact experimental procedures for the human studies I and II are described in Paper II and III, respectively.

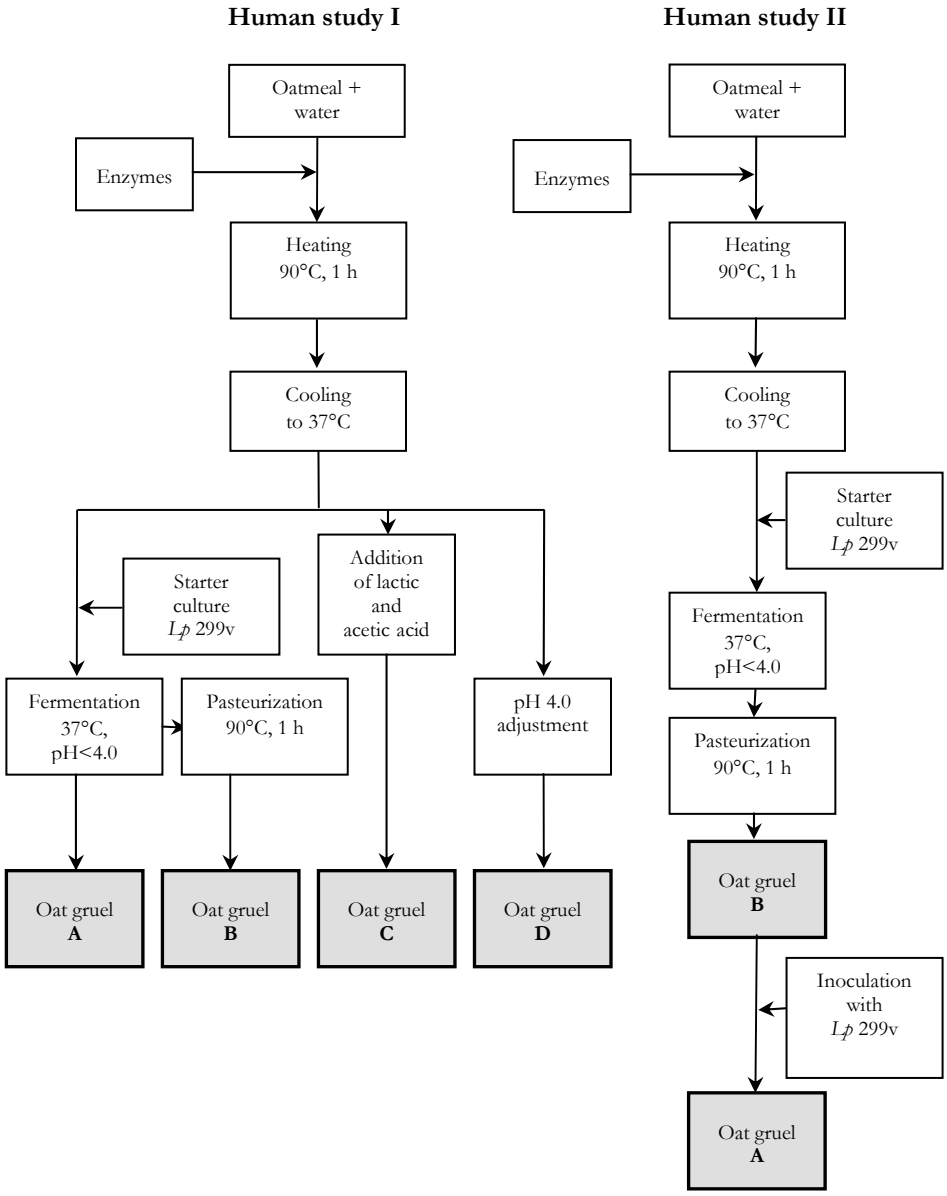

Figure 5 Flow diagrams of the production of the fermented and non-fermented oat gruels for the human studies I and II.

STATISTICAL METHODS

The statistical methods for the analysis of data in the *in vitro* Caco-2 cell study and the human studies I and II are described in detail in the papers I-III.

With the actual measurement of percentage incorporation of iron into the blood from human study II by use of the whole-body countings, these data were used to estimate total absorption of iron in human study I for comparison of iron absorption data between the two studies. The iron absorption data from both human studies were then adjusted to a serum ferritin concentration of 40 μg/L by using the following equation (130):

$$\text{Log } A_{\text{adjusted}} = \log A_{\text{observed}} + \log F - \log 40 \qquad (1)$$

where A is the nonheme iron absorption and F is the serum ferritin concentration.

The basic statistical calculations and analysis, such as calculations of CIs for iron status data of the individuals, and linear regression analyses (with R^2 and *P*-values for deviations of the slope from zero) of iron absorption data and the concentration of lactate in the oat gruels was carried out using Prism (GraphPad Prism version 3.00 for Windows, GraphPad Software, San Diego, CA, USA). Unless otherwise stated values are given as means ± SEM (n = 4) and means are considered significantly different for $P < 0.05$.

RESULTS

THE 3-TIER CACO-2 CELL STUDY

(Paper I)

Iron dialyzability

The amount of iron dialyzed from the peptic digests increased significantly from 13 ± 2% to 24 ± 3% ($P < 0.05$) with increase in lactate from 10 to 200 mmol/L, showing significant correlation with lactate concentration ($R^2 = 0.49$, $P < 0.005$). In contrast, following the pancreatin-bile digestion, lactate (200 mmol/L) caused a significant reduction in the percentage iron dialyzed from 6.9 ± 0.2% to 2.0 ± 0.2% ($P < 0.001$), with an inverse relationship between dialyzable iron and lactate concentration ($R^2 = 0.51$, $P < 0.001$).

Iron uptake

Iron uptake into the cells from the peptic digests was in the region 39 ± 9 to 76 ± 11 pmol/mg protein (1.2-2.8% of the total soluble iron) in the presence of 0-200 mmol/L lactate in the digests with no effect of increasing lactate concentration. During the pancreatin-bile digestion, iron uptake decreased from 228 ± 30 to 8 ± 1 pmol/mg protein ($P < 0.001$) with an increase in lactate concentration from 0 to 200 mmol/L, a decrease from 4.9% to 0.2% of the total soluble iron in the system ($P < 0.001$). The relationship between dialyzable iron and iron uptake from the pancreatic digests was positively and linearly correlated ($R^2 = 0.41$, $P < 0.01$).

Iron transport

From the peptic digests the amount of iron in the basolateral compartment increased with increasing lactate concentrations so that twice as much iron crossed the epithelial layer in the presence of 200 mmol/L lactate than without lactate present. The amount of iron reaching the basolateral side

was proportional to dialyzable iron concentrations (R^2 = 0.87, P < 0.001) but much of this transported iron could be accounted for by paracellular transport as estimated from the rate of ^{14}C-PEG flux from the apical to the basolateral compartment, which was 2.4 ± 0.1%/(h × cm^2) (n = 40), with no difference between peptic and pancreatic digests. Overall 15 ± 2% (n = 20) of the ^{14}C-PEG crossed the dialysis membrane during the peptic incubation period. This compares to 12 ± 1% (n = 20) of the labelled iron added to the food matrix. From the relative concentrations of ^{55}Fe and ^{14}C-PEG the transcellular transport was estimated, and only at 200 mmol/L lactate was there any significant transcellular transport (180 ± 47 pmol/mg protein, P < 0.05). Adding the transcellular transport to the uptake reveals a positive linear relationship between dialyzable iron and total iron uptake during the peptic digestion (R^2 = 0.48, P < 0.01). During the pancreatin-bile digestion phase only 53 ± 6 pmol iron/mg protein (n = 20) was transported to the basolateral side with no effect of lactate. The relative impacts of paracellular and transcellular iron transport were not calculated as only a negligible amount of ^{14}C-PEG was able to cross the dialysis membrane, 0.7 ± 0.1% (n = 20), although 2.6 ± 0.2% (n = 20) of iron was dialyzed.

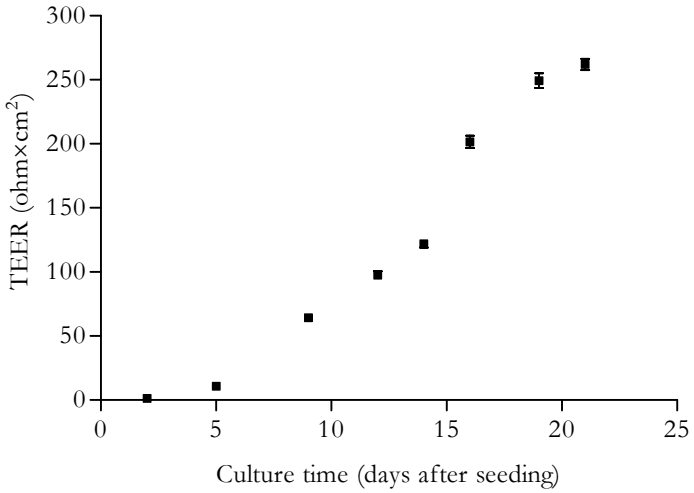

Figure 6 The TEER of the Caco-2 cells as a function of culture time. Values are means ± SEM (n = 48).

The barrier properties of the Caco-2 cell monolayers were assessed by measuring the TEER of the Caco-2 cells during the cell culture period (Figure 6) and the permeability of ^{14}C-mannitol across the Caco-2 cell monolayer in the 3-tier model during the incubation with the peptic and pancreatic digests (Figure 7). The TEER increased slowly and continuously and reached a plateau at 262 ± 4 ohm×cm^2 (n = 48) 21 days after the Caco-2 cells were seeded. The permeability of mannitol across the Caco-2 monolayer was high initially (3.7 ± 0.3 cm/min × 10^{-4} after 10 min, n = 48) and decreased gradually to reach a steady-state after approximately 60 min (1.12 ± 0.06 cm/min × 10^{-4}, mean of 60, 90, and 120 min, n = 3 × 48), with no difference between peptic and pancreatic digests.

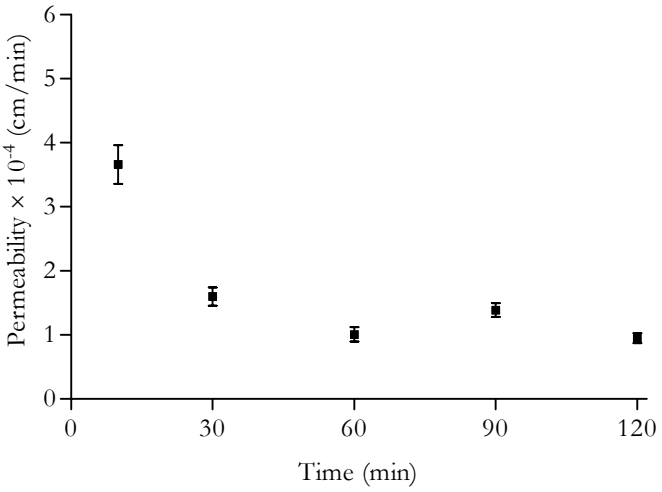

Figure 7 Permeability of ^{14}C-mannitol across the Caco-2 cell monolayer from *in vitro* digested rye bread with increasing amounts of lactate (0-200 mmol/L initial concentration) during the 2 h incubation in the 3-tier Caco-2 cell system. Values are means ± SEM (n = 48).

HUMAN STUDIES

(Paper II and III)

Iron status

The serum ferritin concentrations of the subjects included in the human studies were in the range 12-40 μg/L (Table 2). Six persons in human study I (Paper II) were above the serum ferritin cut-off value for being iron deficient, *i.e.* > 30 μg/L (3). The hemoglobin concentrations were in the range 111-137 g/L (Table 2), and only one person had values slightly below the normale range for healthy Danish women (112.8-161.1 g/L). However, 11 subjects were below the level indicative of anemia as established by WHO, *i.e.* a hemoglobin concentration < 120 g/L for women (9).

Table 2 Iron status of the subjects in human study I and II.

	Age (year)[1]	Hemoglobin (g/L)[1]	Serum ferritin (μg/L)[2]
Human study I (n = 24)	25 (24, 27)	123 (120, 126)	22 (18, 26)
Human study II (n = 18)	23 (21, 24)	124 (121, 127)	20 (18, 22)

[1] Means with 95% CI in parentheses.
[2] Geometric means with 95% CI in parentheses.

Composition of test meals

The levels of the most important components in the test meals in human study I and II are given in Table 3. The total list of analyzed variables is given in Paper II and Paper III.

Within each study there was no difference in the amount of iron and the major inhibitors in the test meals, and the difference in iron and inhibitor content in the test meals between the 2 studies was most likely due to the difference in dry matter in the oat gruels. Consequently, the molar ratios of phytate:iron in human study I and II were similar (12.6 ± 0.6, n = 4, and 12.0 ± 0.5, n = 2, respectively). The concentration of lactate in the test

meals in human study I varied with 19-61%, whereas it was similar in human study II.

Table 3 The content of iron and inhibitors in the test meals and the dry matter and concentration of lactate in the oat gruels in human study I and II.

	Fermented oat gruel with viable *Lp* 299v	Fermented oat gruel with inactive *Lp* 299v	Non-fermented oat gruel with organic acids	Non-fermented oat gruel (pH adjusted)
Human study I (n= 24)				
Nonheme iron (mg)	2.8	2.8	2.8	2.5
Phytate† (mg)	403	393	344	388
Polyphenols‡ (mg)	41	42	42	37
Dry matter (g/100g)	24	24	25	25
Lactate (µmol/g)	110	89	43	61
pH	3.9	4.1	4.0	4.2
Molar lactate:iron ratio	220	178	86	122
Human study II (n = 18)				
Nonheme iron (mg)	1.9	1.9	-	-
Phytate† (mg)	234	237	-	-
Polyphenols‡ (mg)	43	44	-	-
Dry matter (g/100 g)	15	15	-	-
Lactate (µmol/g)	92	99	-	-
pH	3.8	3.8	-	-
Molar lactate:iron ratio	271	292	-	-

† Represents individual inositol tri- to hexaphosphates.
‡ Determined as gallic acid equivalents.

Nonheme iron absorption

The nonheme iron absorption ratios from the different oat gruels are given in Table 4. In the first study only the absorption of nonheme iron into the blood was determined, and there was a significant increase in iron absorption from the fermented oat gruel with the viable *Lp* 299v compared to both the heat-inactivated fermented oat gruel and the non-fermented oat gruel (1.1% vs. 0.5-0.6%, $P < 0.0001$). In the second study total iron absorption was determined from the whole-body retention of ^{59}Fe, and no

difference in absorption between the heat-inactivated fermented oat gruels with and without added viable lyophilized *Lp* 299v was observed (1.4% and 1.3%, respectively). From the relative activity of ^{59}Fe in blood and ^{59}Fe whole-body retention, the percentage of iron absorbed into the blood was determined (86 ± 4%, n = 18). In addition, absorption of ^{55}Fe delivered to the ileum and colon by release from the enterocoated capsules was determined, and this was below the detection limit, *i.e.* < 0.1%.

Table 4 Nonheme iron absorption data from human study I and II.

	Fermented oat gruel with viable¤ *Lp* 299v	Fermented oat gruel with inactive *Lp* 299v	Non-fermented oat gruel with organic acids	Non-fermented oat gruel (pH adjusted)
Human study I (n = 24)				
Fe absorbed into blood (%)†	1.1 (0.8, 1.5)*	0.6 (0.4, 0.7)	0.5 (0.4, 0.7)	0.5 (0.4, 0.7)
Test meal:control meal‡	2.2 (1.7, 2.9)*	1.1 (0.8, 1.4)	1.0 (0.8, 1.3)	-
Calculated Fe absorbed in whole body (%)#†	1.4 (1.1, 2.0)*	0.7 (0.5, 1.0)	0.6 (0.5, 0.9)	0.6 (0.5, 0.8)
Whole-body Fe absorption adjusted to 40 µg/L serum ferritin#†	0.7 (0.5, 1.0)*	0.4 (0.3, 0.5)	0.3 (0.2, 0.4)	0.3 (0.2, 0.4)
Human study II (n = 18)				
Fe absorbed from meal in whole body (%)†	1.4 (0.9, 2.2)	1.3 (0.9, 2.0)	-	-
Test meal:control meal‡	1.1 (0.7, 1.7)	-	-	-
Whole-body Fe absorption adjusted to 40 µg/L serum ferritin#†	0.7 (0.5, 1.1)	0.7 (0.4, 1.0)	-	-
Fe absorbed from enterocoated capsules in whole body (%)†	< 0.1	< 0.1	-	-

¤ Viable active and viable lyophilized lactobacillus in human study I and II, respectively.
† Geometric means of least squares estimates from the mixed linear model analysis with 95% CI in parentheses.
‡ Geometric means of estimates of differences from the mixed linear model analysis with 95% CI in parentheses.
# The whole body retention of iron was calculated from the activity of iron in the blood, based on the anticipation that 86% of the absorbed iron is present in the blood, as calculated from human study II.
* Values are significantly different from all the other values in each row ($P < 0.0001$).

The relation between whole-body iron absorption data adjusted to a serum ferritin concentration of 40 μg/L and the concentration of lactate in the oat gruels in the two human studies is shown in Figure 8. The simple linear regression analysis revealed a positive increase in log(iron absorption) with increase in lactate concentration ($R^2 = 0.57$, $P < 0.001$), which indicates that the nonheme iron absorption is exponentially correlated to the lactate concentration.

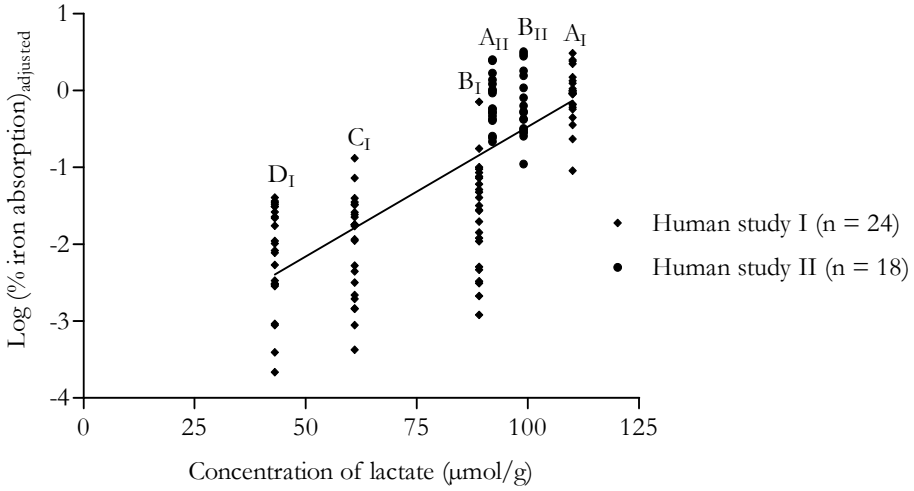

Figure 8 The relation between log (% whole-body iron absorption) and the concentration of lactate in the oat gruels in human study I and II. The whole-body retention of iron in human study I was calculated from the activity of iron in the blood, based on the anticipation that 86% of the absorbed iron is present in the blood, as calculated from human study II, and all data were then adjusted to a serum ferritin concentration of 40 μg/L. The data point at each concentration of lactate represents data from the same test meal, where A are the fermented oat gruels with viable *Lp* 299v, B are the fermented oat gruels with heat-inactivated *Lp* 299v, C is the pH adjusted non-fermented oat gruel, and D is the non-fermented oat gruel added organic acids. I and II denotes human study I and II, respectively. The points represents iron absorption data for each subject, and the line is fitted by linear regression ($R^2 = 0.57$, $P < 0.001$).

DISCUSSION

The investigation of the effect of lactate (0-200 mmol/L) on iron uptake in the *in vitro* digestion/3-tier Caco-2 cell model revealed a positive linear relationship ($R^2 = 0.49$, $P < 0.005$) between the amount of dialyzable iron and lactate concentration after pepsin digestion at pH 2.0 and adjustment of pH to 5.5, whereas there was a negative linear relationship ($R^2 = 0.51$, $P < 0.001$) between the amount of dialyzable iron and lactate concentration after digestion with pancreatin and bile at pH 6.5 in the 3-tier system. Compared to the *in vivo* system the 3-tier Caco-2 cell model is aimed at simulating, the increase in dialyzable iron with increasing lactate concentration after pepsin digestion may reflect an increase in iron bioaccessibility with increasing lactate concentration in the upper duodenum proximal to the sphincter of Oddi, where the pancreatic secretions are excreted. In contrast, with the lower amount of dialyzable iron from the pancreatic digests, which is further reduced with increasing lactate concentration, lactate may decrease the bioaccessibility of iron *in vivo* when moving from the sphincter of Oddi in the duodenum to the upper jejunum.

Iron uptake into the Caco-2 cells after the 2 h incubation with the peptic and pancreatic digests, revealed no effect of lactate on iron uptake after the pepsin digestion phase (39-76 pmol/mg protein), whereas iron uptake decreased after the pancreatin digestion (228 to 8 pmol/mg protein) with increasing lactate concentration (0 to 200 mmol/L), showing positive correlation with iron dialyzability ($R^2 = 0.41$, $P < 0.01$).

The transcellular transport of iron to the basolateral compartment was estimated for the peptic digests only, but these data has to be taken with great caution due to the compromised monolayer with $2.4 \pm 0.1\%/(h \times cm^2)$ transfer of PEG compared to $0.06\%/(h \times cm^2)$ for tight monolayers as obtained with mannitol in similar studies (91). The estimate was based on the assumption that iron and PEG move equally paracellularly across the monolayer, although this may not be a very reliable assumption due to the

large difference in molecular size. As the total iron transport increased with increasing lactate concentration, and the paracellular transport of PEG was constant, there seems to be an increase in active transcellular transport of iron across the Caco-2 cells, probably due to the increase in apical iron concentrations with increase in iron dialyzability, thereby giving a positive linear correlation of total apical iron uptake (uptake and transport) to dialyzable iron ($R^2 = 0.48$, respectively, $P < 0.01$).

The improvement of the Caco-2 cell model to a 3-tier model makes it possible to study the individual steps in dietary iron absorption, and in this study it looks like the overall effect of lactate is directed mainly towards an effect of iron dialyzability and thereby on iron bioaccessibility. With the compromised monolayer, the transport data were not valid for real calculations and interpretation in this study, and were only used as estimates of the directional effect of lactate. As the dialysis of iron was the step mostly affected by lactate the missing transport data probably would not add much to the results of this study, but being an important factor in estimating iron bioavailability by simulating the amount that actually enters the bloodstream and the factor that adds to the earlier Caco-2 cell model described by Glahn *et al.* (112), the actual transport needs to be investigated in more detail.

With the supplemental kinetic study in the 3-tier Caco-2 cell model, the permeability of mannitol across the Caco-2 monolayer was determined. Figure 6 shows that the Caco-2 cells reached a steady-state TEER level at approximately 20 days after seeding, confirming that the cells had differentiated into a monolayer with tight-junction complexes at the day of the experiment. The problem with low dialyzability of the permeability marker was solved by adding mannitol directly to the apical compartment, and the results revealed an initial high permeability, which decreased to reach a steady-state after approximately 60 min ($1.1 \cdot 10^{-4} \pm 0.7 \cdot 10^{-4}$ cm/min). This steady-state permeability is higher than observed in another study ($0.18 \cdot 10^{-4}$ cm/min) with similar growth conditions, but this study was in the normal 2-tier system with the Caco-2 cells grown on 1 cm^2 Transwell membranes with no inserted dialysis membrane (131). The high initial permeability is most likely due to mechanical disturbance, which levels of with time, so that

in future studies the transport rates should be determined from the iron transport to the basolateral compartment at > 60 min after exclusion of monolayers with permeabilities at this stage above the steady-state level.

Furthermore, the split-up of the *in vitro* digestion for investigation of the transfer of iron from both peptic and pancreatic digests revealed that the effect of lactate on iron uptake and transport is dependent on the digestive stage, as the iron uptake was unchanged with increasing lactate concentration for the peptic digests at pH 5.5, whereas it decreased from the pancreatic digests at pH 6.5, similar to findings from pancreatic digestions in other Caco-2 cell studies (67,68). Taking into account the iron transport into the basolateral compartment, lactate seems to increase iron transport only from the peptic digests at pH 5.5. Whether the iron transport actually is steady or decreased from pancreatic digests, simulating unchanged or decreased absorption rates of iron into the circulation *in vivo* with increasing lactate concentrations remains to be established.

In conclusion, the *in vitro* Caco-2 cell study indicates that lactate changes from acting as an enhancer of dietary nonheme iron absorption only very early in the small intestine, *i.e.* in the proximal duodenum, to an inhibitor when mixing of the intestinal content with the pancreatic secretions and a rise in pH. The sphincter of Oddi is only a theoretical set-point to where the pancreatic secretions change the luminal conditions. Taking into account the large variations in buffer capacity of different meals and reflux of the intestinal content with meal type and size, and therefore variations in the pH gradient moving down the duodenum and proximal jejunum, it leaves a very small segment of the small intestine for lactate to increase iron absorption from a meal. On the other hand as most of the dietary iron absorbed mainly is believed to be absorbed in this region (29), the effect of lactate as an enhancer under these circumstances makes it more effective. Furthermore, even though the transit time through this short segment is relatively fast following a meal, the gastric emptying lasts for several hours, and the cells covering the duodenal wall will therefore be exposed to this digested food for several hours.

The organic acids from lactic acid fermented foods have, in addition to the lowering of pH, which leads to activation of endogenous phytases (36,62), and the buffer capacity, which delays precipitation of iron (17), been hypothesized as possible ligands for iron, forming soluble complexes at intestinal pH (17,132). With the indication of a possible enhancing effect of lactate in the most proximal duodenum changing to an inhibiting effect when adding pancreatic enzymes and bile at pH ≥ 6.5 as found in this and other Caco-2 cell studies (67,68), the Caco-2 cell studies has not been able to clearly confirm lactate as an enhancer of iron absorption at intestinal pH, which questions lactate as the major enhancing component in lactic acid fermented foods.

The question is whether the effect of lactate on nonheme iron absorption determined in the *in vitro* Caco-2 cell studies can be extrapolated to humans. Results from human studies describing the effect of lactate and lactic acid fermented foods on nonheme iron absorption are also inconsistent. Lactic acid fermented beer (15) and sauerkraut (16,17), have shown to increase iron absorption, and as the lactic acid bacteria were heat-inactivated before ingestion, lactate has been hypothesized as one of the key compounds responsible for the increased iron absorption in these studies. In the unpaired study by Gillooly *et al.* (16) sauerkraut (160 mg lactate) had the highest iron absorption ratio compared to various vegetable meals containing other organic acids (Six Indian women with geometric mean serum ferritin concentrations of 29 μg/L, SD range 12-70). Derman *et al.* (15) investigated the effect of several characteristics of lactic acid fermented beer on iron absorption in paired designs with both males and females with wide variations in serum ferritin concentrations. With an increase in iron absorption from a lactic acid fermented beer with a lactate content of 1800 mg (13 subjects) compared to the non-fermented oat gruel (2.9% to 6.3%), the effect of lactate was further investigated. In a simple iron solution (pH 2.5) the absorption of iron increased from 3.1% to 12.3% (13 subjects) when acidifying with lactate (720 mg) instead of HCl, but the ability of lactate to counteract the effect of suspended cereal solids in a gruel compared to HCl was less pronounced with an increase from 0.4% to 1.2% (21 subjects). Comparing

lactic acid fermented beer with a gruel pH adjusted to the same pH with lactic acid showed no significant difference in iron absorption (15), but only 6 subjects, which were not of low iron status, were used in this study and it might be a question of to little power to detect any difference in iron replete subjects. The effects of prebiotics on iron absorption have for example shown to be most pronounced in iron-deficient rats (70-72). The simple effect of lactate has been investigated in another study, where no increase in iron absorption was seen when adding 340 mg lactate to rice (13 Indian women with geometric mean serum ferritin concentrations of 27 μg/L, SD range 18-40) (66).

The weak indication from the *in vitro* Caco-2 cell studies that lactate might act as an enhancer of iron absorption only in the very proximal duodenum, and the results from the aforementioned human studies with lactic acid fermented foods, revealed no unequivocal effect of lactate, and the positive effects of lactic acid fermented meals on nonheme iron absorption still needs clarification.

Human study I was aimed at investigating the effect of lactate and other fermentation products compared to an effect of the viable lactic acid bacterium, *Lp* 299v, in a lactic acid fermented food, with exclusion of a simple pH effect. For several reasons the specific characteristics of the four different test gruels were not obtained, which resulted in unsuitable controls for the fermented oat gruel with the viable *Lp* 299v and therefore major limitations of this study. The fermentation of the oat gruel was stopped when reaching pH 4, and the outcome showed, unexpectedly to the production company, to be highly variable from time to time, why the actual concentration of lactic acid in the fermented oat gruel (A) ended up being 2.6 times higher than estimated from earlier productions. The control for the specific organic acids, *i.e.* the non-fermented oat gruel added lactate and acetate (D) was therefore not suitable. Furthermore, there was a significant activity of *Lp* 299v in the fermented oat gruel (A) during storage (4°C) between the two periods, resulting in an increase in the lactate concentration of 19% compared to the heat-inactivated fermented oat gruel (B) within this period, making the heat-inactivated fermented oat gruel less suitable as a control for

the fermentation products including lactate. With the smallest difference in lactate concentration it was though the best control available in this study. The pH adjusted non-fermented oat gruel (C) was suppose to be pH adjusted with HCl, which does not form complexes with iron, but the company producing the oat gruel was not allowed to use this acid in their food production, why their approved L-lactic acid was used. A real control for lactate was therefore not obtained. With the range of test meals actually obtained, *i.e.* four test meals with different concentrations of lactate, the study design changed somewhat from being a design with one test meal with active *Lp* 299v compared to three control meals to being a dose-response study with lactate. With direct comparison of the increase in iron absorption versus the increase in lactate concentration in the fermented oat gruel with viable *Lp* 299v compared to the other oat gruel as discussed in Paper II, it was found to be unlikely that the increase in iron absorption from 0.6 to 1.1% ($P < 0.0001$) was mainly an effect of the organic acids and the viable *Lp* 299v was therefore believed to add significantly to the increased iron absorption.

In human study II the concentrations of lactate were 92 ± 5 μmol/g and 99 ± 6 μmol/g in the fermented oat gruels with viable and inactive *Lp* 299v, respectively. The difference was not significant and, as *Lp* 299v was shown to possess some activity during storage at 4°C in human study I, it is unlikely that the concentration of lactate would decrease from inoculation with the bacteria and during the 24 h storage at 4°C, and the difference was therefore most probably caused by the high viscosity of the oat gruels, which resulted in relative standard deviations of 7% in contrast to < 1% for the standard in the analysis. Furthermore it gives some variation in the determination of pH, and these two factors are probably the explanation for the slightly lower pH in the non-fermented oat gruel added organic acids compared to the pH adjusted non-fermented oat gruel in human study I even though the lactate concentration was higher in the latter. It should though also be taken into account that the non-fermented oat gruel added organic acids also contains acetic acid (3.7 μmol/g). With similar lactate concentration in the two test meals given in human study II, as opposed to

human study I, and with no difference in any of the other parameters analyzed, the two meals only differed in terms of the viable contra heat-inactivated *Lp* 299v. The change in the production process to adding viable lyophilized *Lp* 299v to the heat-inactivated lactic acid fermented oat gruel 24 h before ingestion was therefore successful as regards obtaining equal concentrations of lactate in the product, and also in terms of the amount of viable bacteria (cfu). With no difference in iron absorption between these two meals, an effect of the viable *Lp* 299v was not confirmed in this study. Although the company producing the oat gruels guaranteed the addition of the viable lyophilized bacteria as a good alternative to cold storage of the active fermented oat gruel in terms of obtaining the same active product, this was not the case. Post analysis indicated a lag-phase of the lyophilized bacteria as opposed to the active fermenting bacteria as discussed in paper III, which could possibly explain the lack of effect in this study.

The difference in the content of iron and inhibitors between the test meals in human study I and II, which was due to differences in dry matter content of the oat gruels, *i.e.* 24-25 g/100 g and 15 g/100 g in human study I and II, respectively, is taken into account for when comparing iron absorption values by comparing the molar phytate:iron ratios. These ratios are similar, *i.e.* 12.6 and 12.0, respectively, and the iron absorption ratios are therefore directly comparable between the two studies, as long as they are adjusted a serum ferritin concentration of 40 μg/L. With the lack of whole-body counting in human study I the whole-body retention of iron is though only estimates from the activity in the blood, based on the anticipation that 86% of the absorbed iron is present in the blood, as calculated from human study II. This anticipation is considered reasonable, as earlier studies have shown similar levels with high correlation of ^{59}Fe retention from whole-body countings and from blood samples (127,133). When comparing whole-body iron absorption ratios for the fermented oat gruel with viable *Lp* 299v in human study I and the heat-inactivated fermented oat gruel with added viable lyophilized *Lp* 299v in human study II they were both 1.4%, and even when adjusting to a serum ferritin concentration of 40 μg/L they

were similar (0.7%). This corresponds well with the similar molar phytate:iron ratios in the two studies.

As an effect of *Lp* 299v could not be confirmed in human study II, the effect of lactate was re-studied with the use of data from both human studies. Although it was not possible to do a multiple regression analysis in the mixed linear model, taking into account both serum ferritin, subject, and period effects, to specifically assess the effect of the concentration of the individual organic acids (lactate and acetate) on iron absorption, a simple linear regression analysis revealed a positive correlation between whole-body iron absorption and the lactate concentration in the oat gruels (Figure 8). With a goodness of fit of $R^2 = 0.57$ of the linear regression line there is some degree of correlation between the concentration of lactate and iron absorption. Even when disregarding the oat gruels with the viable *Lp* 299v (A_I and A_{II}), the fermented oat gruel with inactive *Lp* 299v from human study II (B_{II}) adds significantly to the increase in iron absorption with increasing concentration of lactate (Figure 8). The iron absorption values in human study II (A_{II} and B_{II}) are slightly higher than in human study I when comparing with the concentration of lactate in the oat gruels (Figure 8). This could be explained by the higher molar lactate:iron ratio in the oat gruels from human study II, *i.e.* 271 compared to 220 in human study I, which support the hypothesis that lactate is the major determinant of iron absorption in these studies. The human studies described earlier correlates to some extent with these results, as the addition of 340 mg lactate to rice did not increase iron absorption (66), and 720 mg lactate in a gruel only resulted in a minor iron absorption ratio compared to the 1800 mg lactate given in the fermented beer (15). The actual absorption ratios are though not directly comparable due to differences in solid content which is poorly described, and the content of phytate and polyphenols in the meals were not analyzed in these studies.

From the view of the data on Figure 8, an alternative explanation for the effect of lactate could be that a threshold level of approximately 90 μmol/g lactate in the oat gruel was reached, corresponding to a total dose of 810 mg. This is though a relatively high dose that would be hard to reach from

normal meals with lactic acid fermented vegetables or cereals to obtain a significant effect.

In conclusion, the two human studies did not reveal unequivocal results regarding the effect of *Lp* 299v. In human study I there was an indication that viable *Lp* 299v exerted an increase in iron absorption above the effect of the lactate, whereas there was no increase in iron absorption from the fermented oat gruel with viable *Lp* 299v in human study II, in which the control gruel was improved. The production process was different in the two studies, which resulted in the better control, but the question is whether this change also resulted in a change in the activity of the viable *Lp* 299v. In human study I the viable *Lp* 299v were the active fermenting bacteria from the fermented oat gruel, whereas in human study II the viable *Lp* 299v were added from a lyophilized inoculum to the pasteurized fermented oat gruel. Even though the viability of the bacteria were the same in the two studies, as documented from the counts of colony forming units, it could be that the bacteria had not reached a significant level of activity during the 24 h at 4°C before ingestion. This could explain the lack of effect of the viable *Lp* 299v in human study II, as the study only measured the direct effect from single meals.

As *Lp* 299v colonize and are metabolic active the intestine, it is possible that an effect of the bacteria would be more pronounced over a wider period, where a regular intake of *Lp* 299v would leave them to exert their effect on later ingested meals. This would be better clarified by predisposing with *Lp* 299v before doing the single meal study or by a longer intervention study. The bacteria would then be given a better chance of settling and colonizing before measuring iron absorption. The lack of iron absorption in the ileum and colon, as determined in human study II using the enterocoated capsules with radioactive iron, does though not indicate that there would be any significant effect of the *Lp* 299v in the colon as hypothesized in an earlier rat study (44). The potential effect of the colonizing *Lp* 299v would probably still mainly be in the most proximal small intestine, where the bacterium has been shown to colonize, although in a minor degree than more distally (60). Effects of specific dietary components on iron absorption from single

meals are harder to obtain when investigating whole diets. This is visualised by the lack of change in serum ferritin concentrations in intervention studies investigating the effects of components that are considered having major effects on iron absorption, *e.g.* ascorbic acid (134,135), calcium (136), or meat intakes (137). However, due to the actual longer period of time for the bacteria to colonize the intestine and compete with other maybe less organic acid producing bacteria, there is a potential chance of observing an effect of colonizing *Lp* 299v on iron absorption over a longer period of intervention.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Even though human iron absorption and metabolism has been a major research area for decades and important progress has been made in the understanding of iron homeostasis, iron deficiency remains one of the most important nutritional deficiencies worldwide, which is due partly to the poor bioavailability of dietary iron.

Lactic acid bacteria are intimately associated with bacteria involved in food fermentation and probiotics. The capacity of lactic acid bacteria to produce organic acids have linked lactic acid fermented foods to iron absorption, in that their acidifying properties are favourable for the solubility of iron and for activation of endogenous phytases and thereby reduction of the phytate content, and the organic acids may be involved in solubilization of iron in iron-ligand complexes. In addition, the apparent connection between prebiotics and iron would suggest that the fermenting lactic acid bacteria could possibly increase iron absorption.

The major aims of this project was to assess the effect of a viable probiotic bacterium, *Lp* 299v, and the fermentation products, mainly lactate, on non-heme iron absorption from phytate rich foods using an *in vitro* digestion/Caco-2 cell model and from two single meal human studies.

The initial experiment in the *in vitro* digestion/3-tier Caco-2 cell model revealed an effect of lactate mainly on iron bioaccessibility, as the step in the 3-tier Caco-2 cell model that was mostly influenced by lactate was the dialysis of iron to the apical compartment. In the peptic digests (pH 5.5) there was an increase in iron dialyzability with increasing lactate concentrations in the digests, whereas the amount of iron dialyzed decreased in the pancreatic digests (pH 6.5), resulting in a decrease in iron uptake. Taking into account the estimated transcellular transport rates, the study revealed a positive linear correlation between iron dialyzability and iron absorption after both

pepsin and pancreatin-bile digestions. The effect of lactate on iron absorption was though only positive before the addition of pancreatin and bile and a rise in pH, and could possibly be explained by the formation of soluble iron-ligand complexes.

With the set-up of the 3-tier Caco-2 cell model, new parameters were included compared to the Caco-2 model developed by Glahn *et al.* (112), and the effect of refining the model to include these parameters needs thorough investigation. The *in vitro* study included in this thesis is therefore only considered a preliminary study highlighting the possible advantages of the model, *i.e.* the measurement of both dialyzability, uptake, and transport directly from an *in vitro* digested food matrix, with concurrent protection of the cells against the digestive enzymes, and the division of the simulated digestion into both peptic and pancreatic digests.

It is obvious from the *in vitro* Caco-2 cell study that the problems with the data for transport of iron to the basolateral compartment needs to be addressed. The marker for monolayer tightness, PEG, was added to the food matrix before adding this into the digest compartment in the 3-tier model. This resulted in very low values of PEG in the apical compartment due to the low dialyzability. In future studies this marker should preferably be added directly to the apical compartment, as was done in the additional kinetic study, preferably using mannitol, which is smaller than PEG. The effect of pancreatin and bile on the dialyzability would need to be investigated further as it might be that the pancreatin and bile increases the density of the test solution depending on the food matrix by micelle formation, which is of great importance in aspects of molecular diffusion, especially when comparing peptic and pancreatic digests. The kinetic study in the 3-tier Caco-2 cell model revealed that the initial permeability across the monolayer is high, most probably due to mechanical disturbance of the monolayer, resulting in a high initial transport of iron that most likely is not transcellular transport. The transcellular transport rates should therefore be determined from kinetic measurements after approximately 60 min, thereby avoiding calculation of transcellular iron transport assuming equal movement of iron and PEG. Furthermore, due to the higher steady-state permeability of man-

nitol across the monolayer than has been found in other studies (131), the effect of inserting the dialysis membrane in the system on the permeability across the Caco-2 cell monolayer is needed, preferably with simple solutions of iron to begin with.

The area of the dialysis membrane in the system is quite small compared to the area of the cell monolayer (1 cm^2 versus 4.7 cm^2), and more equal areas would be preferable for better utilization of the digested food matrix.

As regards the specific study, even though there were four replicates of each sample, all samples were applied to the same passage of the Caco-2 cells and should preferably have been replicated in different passages. Furthermore, the experiments were carried out with a pH in the basolateral compartment similar to that in the apical compartment, *i.e.* pH 5.5 and 6.5 for the peptic and pancreatic digests, respectively. This was done to avoid a pH gradient across the monolayer, but as the basolateral compartment should simulate the conditions in the circulation *in vivo*, the pH should in future studies be physiological, *i.e.* pH 7.4, preferably also with apo-transferrin to provide a sink for iron to bind to upon transfer and serum albumin for better simulation of the blood. When trying to simulate physiological conditions it would be relevant to consider addition of the recently identified regulatory molecule, hepcidin, to the basolateral compartment as well, depending on the desired iron status of the cells. The *in vivo* regulation is though a very hard task to handle *in vitro*, and basic mechanistic studies would be required before general use of these regulatory molecules in bioavailability studies.

Whereas the results from human study I revealed a significant increase in nonheme iron absorption from a lactic acid fermented oat gruel that seemed to be caused by a specific effect of the viable *Lp* 299v more than an effect of the organic acids produced, the results from human study II did not confirm this effect. Furthermore, no absorption of iron from either of the oat gruels was observed in the ileum and colon. In human study II the control gruel without added viable lyophilized *Lp* 299v was similar to the test gruel with the viable *Lp* 299v in the content of iron, lactate, other organic acids, and inhibitors of iron absorption, and therefore a good control

for the added viable lyophilized bacteria as opposed to the controls in human study I, the pasteurized fermented oat gruel and the non-fermented oat gruels, differed significantly in the content of lactate. When comparing all test gruels from human study I and II, there seems to be an exponential correlation between the nonheme iron absorption and the concentration of lactate in the oat gruels, indicating that lactate is the major direct determinant of iron absorption from lactic acid fermented meals. The change in the production process in human study II to obtain a better control gruel for lactate may though have influenced the results in that in human study II the viable bacteria were added as lyophilized viable *Lp* 299v to the heat-inactivated lactic acid fermented oat gruel, whereas in human study I the viable bacteria were the metabolic active *Lp* 299v from the fermentation process.

These two single meal studies indicate that lactate rather than the viable *Lp* 299v is responsible for the increase in nonheme iron absorption from a single meal, although a possible effect of the metabolic active *Lp* 299v on iron absorption in the proximal small intestine still needs clarification.

Therefore, to follow up on these results a more specific investigation of the difference in the activity of *Lp* 299v from a fermented oat gruel with viable *Lp* 299v versus viable lyophilized *Lp* 299v added to a heat-inactivated lactic acid fermented oat gruel would be required to better determine whether active lactic acid bacteria do contribute to an enhanced iron absorption from phytate rich meals. This would include *in vitro* digestion of the two fermented oat gruels with the viable bacteria with pepsin at pH 2 for 2-4 h, followed by an activity test of the bacteria.

If the viable lyophilized *Lp* 299v added to the heat-inactivated lactic acid fermented oat gruel has a lag-phase before reaching the activity level as the bacteria from the active fermented oat gruel, the results from human study I may indicate that there is a potential for active *Lp* 299v in a fermented oat gruel to increase nonheme iron absorption. With no absorption of iron in the ileum and colon in human study II, this effect would most likely be in the proximal small intestine, possibly by local organic acid production or

maybe by stimulating the gene expression of DMT1 in the enterocytes. With this indication studies on the effect of intake of active *Lp* 299v with whole diets is warranted. A 4-5 days intervention trial using radioisotopes to study the effect of active *Lp* 299v on nonheme iron absorption from a whole diet is suggested, and also long-term intervention studies measuring the effect on iron status. Due to the time period for *Lp* 299v to colonize the intestine, another approach would be to measure iron absorption from meals or whole diets after one week of daily administration of the viable *Lp* 299v. The local effect of the active *Lp* 299v in the intestinal mucosa would then be determined. This would though require iron to be absorbed distal to the duodenum as this is where *Lp* 299v colonizes, as the activity of *Lp* 299v as they move along the duodenum is then not measured. As no non-heme iron absorption occurred in the ileum and colon from the single meal in human study II, this seems unlikely, although it is possible that giving the bacteria time to ferment in the colon before measuring iron absorption would increase the local production of organic acids and decrease the pH. It is though unlikely that it would add a significant amount of iron to the total amount absorbed from a meal.

In conclusion, the *in vitro* study described in this thesis adds to the few studies investigating the effect of lactate on iron uptake in an *in vitro* Caco-2 cell model, with addition of the simulated digestion pre- and post the Sphincter of Oddi, and insight into both dialyzability, uptake, and transfer across the Caco-2 cells, revealing an effect of lactate mainly on bioaccessibility. Furthermore, the two single-meal studies add to the human studies with lactic acid fermented foods in that the effect of the viable lactic acid bacteria, in this case *Lp* 299v, was separated from the effect of lactate, revealing an effect of lactate probably more than an effect of the viable bacteria, with no absorption of iron occurring in the colon.

In conclusion, lactic acid fermentation of vegetables and cereals seems to be a favourable process for increasing the bioavailability of iron from foods that are high in phytate, and future studies should reveal whether it is advisable to avoid heat-treatment of such products for optimal utilisation of the lactic acid bacteria or if it is a question of maximal production of organic

acids (within the limit of being eatable) in the product before ingestion. This is of special perspective for developing countries, where lactic acid fermentation still is applied for food preservation. Examples of such lactic acid fermented foods are fermented spinach and sorrel, brined olives, sauerkraut, salted gherkins, sourdough, Nigerian ogi (made from maize or sorghum), Ethiopian Koncho (made from starch from *Ensete ventricosum*) and sourdough (made from tef and cassava). Regarding the Western countries, only Northern Europe have been used to consuming lactic acid fermented foods up to modern time, but fermented food products in terms of probiotic foods are now gaining interest in the Western countries, and could potentially be the future modified foods that contributes to an increased iron absorption from foods with otherwise low iron bioavailability.

ACKNOWLEDGEMENTS

This work was made possible by the help and encouragement of all my colleagues, friends, and family.

Firstly, I would like to express my most sincere gratitude to my supervisors, *Klaus Bukhave* and *Inge Tetens*, for their very expert and inspiring coaching, for their unstinting encouragement, and for providing all the help and support that has been instrumental in the forming of this thesis. A special thank goes to Klaus for sticking it out from start to finish – there are only two of our kind! Also special thanks to my former supervisors, *Marianne Henriksen* and *Brittmarie Sandström*, for giving me the opportunity to work on this PhD project.

I am grateful to all *the women volunteers* for participating in the human studies.

I have greatly appreciated the friendship of all my colleagues at the Department of Human Nutrition. Special thanks go to *Pia L. Madsen*, *Inge Rasmussen*, *Hanne L. Petersen*, *Leif S. Jakobsen, and Berit Hoielt* for their skilful technical assistance. I wish to thank *Claude Mona* for guiding me through the all the regulations and forms that come with being employed at KVL, and my M.Sc. candidates *Laila Sjøltov* and *Seema Suchdev* for their invaluable practical help and great sense of humor. My work and travel mates, *Sussi B. Bæch*, *Lisbeth Grinder-Pedersen*, *Dorthe Sørensen*, and *Camilla Hoppe*, are thanked for great scientific and non-scientific discussions - that will be greatly missed.

I am grateful to the research group of *Susan J. Fairweather-Tait* at The Institute of Food Research, Norwich, UK, who so kindly accommodated my visit. I would like to thank *Elizabeth K. Lund* in particular, for kindly sharing her scientific expertise with me.

Also, I would like to thank *Ann-Sofie Sandberg* for hosting my visit to The Department of Food Science, Chalmers University of Technology, Göteborg, Sweden. Special thanks go to *Annette Almgren* for introducing me to

their research facilities and for carrying out the phytate analyses for the human studies.

I thank *Hanne Frøkiær* and *Hanne Risager* from BioCentrum, The Technical University of Denmark, for introducing me to the world of Caco-2 cells and for letting me use their cell culture facilities.

Probi AB, Lund, Sweden, is thanked for delivering the oat gruels for the human studies.

The people at The Department of Clinical Physiology and Nuclear Medicine, Rigshospitalet, especially *Susanne Svalling* and *Liselotte Højgaard*, are thanked for doing the whole-body countings.

From The Department of Food Chemistry and Nutrition, The Danish Institute for Food and Veterinary Research, I thank *Pia Knuthsen* for doing the polyphenol analyses.

Thank you to all my friends, and especially *Mette Pierri*, *Nanna Stensler-Thomsen* and *Cathrine L. Bak*, whose inspiration from 'outer space' is a relief when my mind becomes rusty with iron research.

Special thanks go to *my family* for support and encouragement, and for teaching me to always look positively at things.

Finally, *Jens*, thank you - for being you, loving me, and for our children, *Emil* and *Sofie*. You are all the world to me!

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