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# BIOFORTIFICATION: OPTIMIZING IRON ABSORPTION FROM BEANS AND OTHER STAPLE FOODS

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## Abbreviations

| | |
|---|---|
| AACC | American Association of Cereal Chemists |
| ACF | Aberrant crypt foci |
| CBG | Cytosolic beta- glucosidase |
| CGIAR | Consultative Group on International Agricultural Research |
| CHI | Chalcone isomerase |
| CHS | Chalcone synthase |
| CIAT | International Center for Tropical Agriculture |
| DALYs | Disability-Adjusted Life Years |
| Dcybt | Duodenal cytochrome B |
| DMT1 | Divalent cation transporter 1 |
| DNA | Deoxyribonucleic acid |
| DP | Degree of polymerization |
| DRC | Democratic Republic of the Congo |
| EDTA | Ethylenediaminetetraacetic acid |
| EGCG | Epigallocatechin-3-gallate |
| FAO | Food and Agriculture Organization |
| Fe | Iron |
| GAE | Gallic acid equivalents |
| GALT | Gut associated lymphoid tissue |
| GC | Gas chromatography |
| G x E | Genetic and Environment |
| GLUT | Sodium independent glucose transporter |
| GMO | Genetically modified organism |
| GSE | Grape seed extract |
| HYV | High yield varieties |
| IBD | Inflammatory bowel disease |
| IBS | Irritable bowel syndrome |
| ID | Iron deficiency |
| IgA | Immunoglobulin A |
| IL | Interleukin |
| IP6 | Myo- inositol- 1, 2, 3, 4, 5, 6- hexakisphosphate |
| IRRI | International Rice Research Institute |

| | |
|---|---|
| Lpa | Low phytic acid mutant |
| LPH | Lactase phlorizin hydrolase |
| PA | Phytic acid |
| PAL | Phenylalanine ammonia- lyase |
| PP | Polyphenols |
| PP | Peyer's patches |
| PPO | Polyphenol oxidase |
| QTL | Quantitative gene loci |
| RNA | Ribonucleic acid |
| SCFA | Short chain fatty acids |
| SGLT1 | Sodium- dependent glucose transporter |
| TAL | Tyrosine ammonia- lyase |
| TfR | Transferrin receptor |
| USDA | United States Department of Agriculture |
| WHO | World Health Organization |
| Zn | Zinc |

# Summary


## Background

Micronutrient deficiencies are a major cause of malnutrition affecting more than two thirds of the world's population, and it is estimated that iron deficiency remains the most common micronutrient deficiency worldwide. Iron deficiency affects all population groups even though children and women of reproductive age are particularly vulnerable. Low iron status is particularly a problem in populations subsisting on plant-based diets that are low in animal tissue and high in iron absorption inhibitors and therefore low in bioavailable iron.

Biofortification, the process of increasing the content and/or bioavailability of essential nutrients such as iron in crops by traditional plant breeding and genetic engineering, is a promising approach to combat micronutrient deficiencies. By definition, for biofortification to be successful it is not sufficient to increase iron content in plants only. Rather it is also necessary to increase iron bioavailability, by either decreasing the level of absorption inhibitors or by increasing the level of absorption enhancers.

One way to increase iron bioavailability from staple crops such as beans is to decrease the level of phytic acid (PA) and polyphenols (PP), which have been shown to be strong inhibitors of absorption in the human body. Another approach might be to increase the level of inulin, a potential enhancer of iron absorption present in some cereal grains.

## Aim

With the overall aim of increasing the intake of bioavailable iron from beans and other staple crops by plant breeding strategies, this project assessed the impact of iron biofortified beans on human iron absorption and evaluated the relative importance of PP and PA on iron bioavailability from beans in humans. Moreover, the project evaluated if the consumption of inulin is beneficial for iron absorption.

**Design**

*Manuscript 1 (studies 1-6):* The dose dependent effect of PP on human iron absorption was investigated in the first three iron absorption studies. Different amounts of bean hulls, as the source of PP were added to a non-inhibitory test meal and iron absorption was assessed by stable iron isotope techniques.
The inhibitory effect of PP and PA in beans was investigated either individually or combined (studies 4-6). Since PP are mainly present in the bean hull and PA exclusively in the cotyledon, beans were served either whole, dehulled, dephytinized or dehulled and dephytinized. The test meals were given in the form of sweetened, homogenized bean porridge. In study 4, the influence of bean PP on iron absorption in the presence of PA was evaluated by comparing iron absorption from beans with and without hulls; in study 5, the combined impact of PP and PA was investigated by comparing iron absorption from whole beans with dehulled, dephytinized beans and in study 6 the influence of PP on iron absorption in the absence of PA was evaluated by comparing dephytinized beans with dephytinized, dehulled beans.

*Manuscript 2 (studies 7-9):* The three iron absorption studies were made in Rwandese women (20 per study) of low iron status. Studies 7 and 8 compared iron absorption from high and low PP beans, similar in PA and iron, fed as a bean puree in a double meal design (study 7) or fed with rice and potatoes in a multiple meal design (study 8). Study 9 tested the performance of a biofortified high iron bean. Iron absorption from high and normal iron beans (9.1 or 5.2 mg Fe/100 g bean) with similar PP levels and a similar PA: iron molar ratio fed with either 170 g potatoes or 40 g rice (dry weight) was compared in a multiple meal design.

*Manuscript 3 (study 10):* The impact of inulin on human iron absorption and gut microbiota was studied in 35 subjects in a randomized crossover design. Participants received an inulin-oligofructose mixture or placebo in a random fashion in a first or second test period, given in divided doses (6g) after each meal (breakfast, lunch, dinner). Each test period lasted for 4 weeks, separated by a 2 week wash out period. To measure iron absorption Fe compounds were labeled with $^{57}Fe$ and $^{58}Fe$, respectively, and added to a test meals served at the end of the third week of each four week feeding period.

## Results

*Manuscript 1 (studies 1-6):* Iron absorption was lowered by 14 % with 50 mg PP ($p< 0.05$); and by 45 % with 200 mg PP ($p< 0.001$), whereas the lowest PP concentration (20 mg) had no effect on iron absorption.
Mean iron absorption from whole bean porridge was 2.5 %. PP and PA removal increased absorption 3.6 fold ($p< 0.001$) and removal of PP from dephytinized porridge increased absorption 2 fold ($p< 0.001$). Between study comparisons indicated that dephytinization did not increase iron absorption in the presence of PP; but in their absence absorption increased 4.4 fold ($p< 0.001$).

*Manuscript 2 (studies 7-9):* Mean fractional iron absorption from the low PP bean meal was 27 % higher than absorption from the high PP bean meal ($P< 0.005$) in study 7.
In contrary to study 7 mean fractional iron absorption of participants in study 8 consuming the high and low PP bean with rice or potatoes was about 7 % from both meals and did not differ.
Mean iron absorption from the biofortified high iron bean was 60 % lower than from the normal iron bean ($p< 0.001$), which resulted in total absorbed iron from the high and normal iron bean meals not being significantly different.

*Manuscript 3 (study 10):* Mean fractional iron absorption in the inulin phase was 15.2 % and did not differ significantly from iron absorption in the placebo phase (13.3 %). Inulin significantly reduced fecal pH ($P< 0.001$) and significantly increased bifidobacteria population ($P< 0.001$) and lactate ($P< 0.001$). Inulin had no impact on fecal short chain fatty acid (SCFA) profile.

## Conclusion

1) The present findings clearly showed that bean PP have a dose dependent effect on human iron bioavailability in the absence of PA (studies 1-3). They furthermore demonstrated that both PA and PP inhibit iron absorption from beans within the bean matrix, whereas their inhibitory effect seemed not to be additive (studies 4-6). However, a moderate impact of bean PP on iron absorption was observed when

beans were served in a double meal design in a bean consuming population (study 7). This effect was not seen any more when beans were administered with rice and potatoes in a multiple meal design (study 8). These differing results might be due to the often observed overestimation of the effect of inhibitors in single meal studies. It is also possible that other meal components weakened the impact of PP and PA on iron absorption by reducing or chelating effects or simply by diluting the present inhibitors.

The results of study 9 raised the general question if a biofortified bean has the potential to significantly improve the iron status of a bean consuming population. The high iron bean in that study did not provide a greater amount of bioavailable iron when compared to a non-biofortified normal iron bean. The results indicate that the presence of PP and PA prevents the absorption of an additional amount of iron from high iron beans. This leads to the conclusion that, for beans to be a potential vehicle for iron biofortification, they must be high in iron and low in PP and PA.

2) Although inulin and oligofructose exhibited a positive effect on iron absorption in animals the study was not able to show the same effect in humans. Iron absorption was slightly higher during inulin consumption, albert not significantly. It is assumed that inulin affects colonic iron absorption, which is rather low compared to duodenal iron absorption. Therefore an inulin induced change in colonic absorption might have remained undetected. However the bifidogenic effect of inulin was clearly demonstrated.

# Zusammenfassung


## Hintergrund

Mehr als 30 % der Weltbevölkerung leidet unter Mikronährstoffmangel und es wird angenommen, dass Eisenmangel weltweit der am häufigsten auftretende Mangel ist. Eisenmangel betrifft alle Bevölkerungsgruppen, wobei vor allem Kinder als auch Frauen im gebärfähigen Alter zu den meistgefährdeten Gruppen zu zählen sind. Eisenmangel ist vor allem dort ein Problem, wo die Nahrung der Menschen auf einseitiger Pflanzenkost basiert. Diese Kost besteht gewöhnlich nur zu einem geringen Anteil aus Fleisch und weist einen hohen Gehalt an Stoffen auf, die die Aufnahme von Eisen hemmen.

Biofortifizierung, das Erhöhen der Konzentration und/oder der Bioverfügbarkeit von essentiellen Nährstoffen in Feldfrüchten durch traditionelle Züchtung oder Gentechnik, ist ein neuer, vielversprechender Ansatz um Mikronährstoffmängeln entgegenzuwirken. Um die Erfolgsaussichten von Biofortifizierung zu optimieren, muss neben einer Erhöhung der Mikronährstoffkonzentration auch der Gehalt an Inhibitoren minimiert oder der Gehalt an Förderern der Eisenaufnahme maximiert werden.

Eine Möglichkeit, die Eisenbioverfügbarkeit von Grundnahrungsmitteln, wie z.B. Bohnen zu erhöhen, ist die Reduktion von Phytinsäure und Polyphenolen, beides starke Inhibitoren der Eisenaufnahme. Ein weiterer Ansatz wäre die Konzentrationserhöhung von Inulin, ein potentieller Förderer der Eisenaufnahme, vornehmlich zu finden in verschiedenen Getreidesorten.

## Ziel

Mit dem allumfassenden Ziel die Einnahme von Eisen aus Bohnen und anderen Grundnahrungsmitteln durch Strategien der traditionellen Pflanzenzüchtung zu erhöhen, befasste sich diese These mit dem Einfluss von biofortifizierten Bohnen auf die menschliche Eisenaufnahme und evaluierte den relativen Einfluss von Polyphenolen und Phytinsäure auf die Eisenbioverfügbarkeit von Bohnen im

Menschen. Zudem wurde ermittelt, ob Inulin der Eisenaufnahme im Menschen förderlich ist.

**Studiendesign**

*Manuskript 1 (Studien 1-6):* In den Studien 1 bis 3 wurde der dosisabhängige Effekt von Polyphenolen auf die menschliche Eisenaufnahme untersucht. Verschiedene Mengen an Bohnenhüllen, die Quelle von Polyphenolen wurden einer nicht hemmenden Testmahlzeit zugesetzt und die Eisenabsorption wurde mittels stabiler Eisenisotopen Technik erfasst. In den Studien 4 bis 6 wurde der inhibierende Effekt von Polyphenolen und Phytinsäure, sowohl im Einzelnen als auch in Kombination untersucht. In Studie 4 wurde der Einfluss von Polyphenolen im Beisein von Phytinsäure, durch den Vergleich von Bohnen mit und ohne Bohnenhülle, auf die Eisenaufnahme, evaluiert. Studie 5 hatte zum Ziel, den kombinierten Einfluss von Polyphenolen und Phytinsäure auf die Eisenaufnahme zu untersuchen. Als Testmahlzeiten dienten ganze Bohnen, welche mit dephytinisierten Bohnen ohne Hülle verglichen wurden. In Studie 6 wurde dann der Einfluss von Polyphenolen in Abwesenheit von Phytinsäure auf die Eisenaufnahme evaluiert. Hierzu wurde die Absorption von dephytinisierten Bohnen mit dephytinisierten Bohnen ohne Hülle verglichen.

*Manuskript 2 (Studie 7-9):* Die drei Eisenisotopenstudien wurden in eisendefizienten ruandischen Frauen mit niedrigem Eisenstatus durchgeführt (20 pro Studie). In Studien 7 und 8 wurde die Eisenaufnahme von Bohnen mit hohem und niedrigem Polyphenolgehalt, sowie gleicher Phytinsäure- und Eisenkonzentration ermittelt. Die Bohnen wurden entweder als Bohnenpüree gereicht (Studie 7; "double meal design") oder mit Reis/Kartoffeln über mehrere Tage (Studie 8; "multiple meal design"). In Studie 9 wurde die Effektivität einer biofortifizierten Bohne mit hohem Eisengehalt getestet. Die Studie hatte ein "multiple meal design" und es wurde die Eisenabsorption von einer biofortifizieren Bohne mit hoher Eisenkonzentration zu einer Bohne mit normaler Eisenkonzentration verglichen (9.1 und 5.2 mg Eisen/ 100 g Bohnen). Die Bohnen wiesen den gleichen Gehalt an Polyphenolen und das gleiche molare Verhältnis von Phytinsäure zu Eisen auf. Die Testmahlzeiten bestanden aus Bohnen und 40 g Reis oder 170 g Kartoffeln.

*Manuskript 3 (Studie 10):* Der Einfluss von Inulin auf die menschliche Eisenaufnahme wurde in einer randomisierten, doppelt blinden Crossover-Studie untersucht. Die Teilnehmer erhielten entweder Inulin oder Placebo, 3 x täglich circa 6 g zu den Mahlzeiten. Jede Testphase hatte eine Dauer von 4 Wochen, separiert durch eine zweiwöchige Auswaschphase. Um die Eisenaufnahme zu messen, wurden mit Eisenisotopen versetzte Testmahlzeiten am Ende der dritten Woche jeder Testphase verabreicht.

**Resultate**

*Manuskript 1 (Studien 1-6):* 50 mg Polyphenole reduzierten die Eisenaufnahme um 14 % ($p < 0.05$), wobei 200 mg Polyphenole die Eisenabsorption um 45 % verringerten. Die niedrigste Polyphenolkonzentration (20 mg) hatte keinen Einfluss auf die Eisenaufnahme.
Die durchschnittliche Eisenabsorption von dem Brei aus ganzen Bohnen betrug 2.5 %. Durch das Entfernen von Polyphenolen und Phytinsäure wurde die Absorption 3.6-fach erhöht ($p < 0.001$) und das Entfernen von Polyphenolen von zuvor dephytinisiertem Bohnenbrei erhöhte die Absorption zweifach. Vergleiche zwischen den Studien zeigten, dass Dephytinisierung in der Gegenwart von Polyphenolen nicht zu einer Erhöhung der Eisenaufnahme führte, aber in ihrer Abwesenheit stieg die Absorption um das 4.4-fache an ($p < 0.001$).

*Manuskript 2 (Studie 7-9):* Die durchschnittliche Eisenabsorption von der Bohnenmahlzeit, welche die Bohne mit geringer Polyphenolkonzentration enthielt war 27 % höher als von der Mahlzeit mit der Bohne die eine hohe Polyphenolkonzentration aufwies ($P < 0.005$).
Die durchschnittliche Eisenaufnahme, sowohl von der Bohne mit niedrigem als auch von der Bohne mit hohem Polyphenolgehalt war ungefähr 7 % und somit nicht signifikant unterschiedlich.
Die durchschnittliche Eisenabsorption von der Bohne mit hohem Eisengehalt war 60 % niedriger als von der Bohne mit niedrigem Gehalt ($P < 0.001$), was in der gleichen Menge an absolut aufgenommenem Eisen resultierte.

*Manuskript 3 (Studie 10):* Die durchschnittliche Eisenaufnahme während der Inulin Konsumierung betrug 15.2 % und unterschied sich nicht signifikant von der Eisenaufnahme in der Placebophase (13.3 %). Die Gabe von Inulin führte zu einer signifikanten Reduktion des pH ($P< 0.001$), zu einer signifikanten Erhöhung der Bifidobakterienpopulation und Laktat ($P< 0.001$). Inulin hatte keinen Einfluss auf die Konzentration von kurzkettigen Fettsäuren.

**Schlussfolgerungen**

Die durchgeführten Untersuchungen zeigen eindeutig, dass Bohnenpolyphenole einen dosisabhängigen Einfluss auf die Eisenaufnahme in Abwesenheit von Phytinsäure haben (Studien 1-3). Weiter konnte gezeigt werden, dass sowohl Phytinsäure als auch Polyphenole die Eisenaufnahme von Bohnen innerhalb der Bohnenmatrix beeinflussen, wobei dieser Effekt nicht additiv ist (Studien 4- 6). In einer Studie, die in einer bohnenverzehrenden Population durchgeführt wurde ("double meal design"), konnte ein moderater Effekt von Bohnenpolyphenolen auf die Eisenaufnahme beobachtet werden (Studie 7). Wurden die Bohnen allerdings mit Reis und Kartoffeln serviert ("multiple meal design"), konnte dieser Effekt nicht mehr nachgewiesen werden (Studie 8). Die unterschiedlichen Ergebnisse von Studie 7 und 8 könnten durch die schon des Öfteren beobachtete Überbewertung des Effektes von Inhibitoren und Förderern der Eisenaufnahme in Studien mit "single meal design" aufgetreten sein. Weiterhin könnten auch andere in den Mahlzeiten enthaltene Substanzen, durch Eisenreduktion, Chelatbildung mit Eisen oder Verdünnung der Inhibitoren, den Einfluss von Polyphenolen und Phytinsäure gemindert haben.
Aufgrund der Resultate von Studie 9 muss generell das Potential biofortifizierter Bohnen, den Eisenstatus in einer bohnenkonsumierenden Bevölkerung zu verbessern, in Frage gestellt werden. Die verwendete Bohne mit erhöhtem Eisengehalt dieser Studie stellte keine grössere Menge an bioverfügbaren Eisen zur Verfügung. Die Resultate der Studie weisen darauf hin, dass die Gegenwart von Polyphenolen und Phytinsäure die Aufnahme von zusätzlichem Eisen von der Bohne mit hohem Eisengehalt verhindert. Damit eine biofortifizierte Bohne das Potential hat sich positiv auf den Eisenstatus auszuwirken, muss sie sowohl einen hohen Eisengehalt, als auch gleichzeitig auftretende geringe Konzentrationen von Polyphenolen und Phytinsäure aufweisen.

Obwohl sowohl Inulin als auch Oligofruktose einen positiven Effekt auf die Eisenaufnahme von Tieren zeigten, war es uns nicht möglich denselben Effekt im Menschen zu zeigen. Die Eisenaufnahme während der Konsumierung von Inulin war leicht erhöht, jedoch war der Unterschied zur Eisenaufnahme während der Konsumierung von Placebo nicht signifikant. Es wird angenommen, dass Inulin die Eisenaufnahme im Dickdarm beeinflusst, diese jedoch im Vergleich zur Aufnahme im Duodenum eher gering ist. Deshalb besteht die Möglichkeit, dass eine von Inulin induzierte Verbesserung der Eisenaufnahme im Dickdarm nicht detektiert wurde. Es konnte jedoch der „bifidogene Effekt“ von Inulin gezeigt werden.

# Introduction

Micronutrient deficiencies are a major public health problem affecting more than 30% of the world's population. It is estimated that iron deficiency remains the most common micronutrient deficiency worldwide. Young children and women of reproductive age are particularly vulnerable even though it affects all population groups. Even milder forms may adversely influence the cognitive performance, behavior, and physical growth of infants, preschool and school-aged children as well as the physical capacity and work performance of adults.

Several public health strategies exist to prevent or reduce micronutrient deficiencies. However, there is still need for new interventions since the existing have not been universally successful. Biofortification, the development of crops with increased concentration of bioavailable nutrients, is a new approach to combat micronutrient deficiencies. This strategy enables plant breeders to distribute seeds, which efficiently accumulate minerals such as iron, to the whole population including low-income households in rural areas. Rice, wheat, maize, the common bean and cassava are the main targeted crops of biofortification programs and initiatives, potentially delivering iron, zinc and vitamin A to people in developing countries.

However, plant-based diets are low in animal tissue and high in iron absorption inhibitors and therefore low in bioavailable iron. Thus, for biofortification to be successful it might not be sufficient to increase iron concentration of targeted crops, but also necessary to increase iron bioavailability by decreasing the level of absorption inhibitors or increasing the level of absorption enhancers.

One way to increase iron bioavailability is to decrease the level of PA and PP, which have been shown to be the two major inhibitors of non-heme iron absorption in cereals and legumes. Increasing the concentration of inulin, which is present in considerable amounts in wheat might be a further strategy to make biofortification more successful. Inulin has been shown to enhance iron absorption in animals, but studies attempting to show the same effect in humans have so far failed.

With the overall aim of increasing the intake of bioavailable iron from beans by plant breeding strategies, the first part of the project evaluated the relative importance of PA and PP on iron bioavailability from beans in humans. In the second part the performance of several bean varieties was tested in Rwanda, the country with the highest bean consumption worldwide. The third part of the project investigated,

whether inulin could be a useful compound in biofortification to increase iron absorption from wheat and other staple crops.

The literature review proceeding the original research focuses on the potential of biofortification as a new approach to reduce micronutrient deficiencies, transgenic and traditional plant breeding approaches and the different crops currently undergoing the biofortification process with focus on common beans, PP as well as inulin.

The part describing original research is structured into 3 manuscripts:

Manuscript 1: Polyphenols and phytic acid contribute to the low iron bioavailability from common beans in young women.

Manuscript 2: The potential of the common bean (Phaseolus vulgaris) as a vehicle for iron biofortification.

Manuscript 3: Inulin changes gut microflora but does not increase iron absorption in women with low iron status.

# 1 Biofortification

Biofortification, a new approach to combat micronutrient deficiencies, is defined as the process of increasing the concentration and/or bioavailability of essential elements in the edible part of the plant by traditional plant breeding or genetic engineering (White and Broadley 2005). By definition, the focus of plant breeders and biofortification initiatives is on breeding crops with a high density and increased bioavailability of nutrients.

The HarvestPlus program is globally leading the development of biofortified crops and works with more than 200 agricultural and nutrition scientists around the world.

This chapter will discuss on the potential of biofortification as a tool for fighting micronutrient malnutrition in developing countries. The focus will be on 1) the different steps which are to be taken to successfully implement biofortification programs; 2) the current stage and progress of biofortification, and 3) beans as a suitable crop for iron biofortification.

## 1.1 Biofortification as a tool to combat micronutrient deficiencies

Micronutrient deficiencies are also referred to as "hidden hunger" since they are often not clinically visible, so that people might suffer from them without being aware. Iron, vitamin A, iodine and zinc deficiencies are among the world's most serious health risk factors and substantially contribute to the global burden of disease. It has been estimated that micronutrient deficiencies affect more than 2 billion people. They lead to low work productivity, permanent impairment of cognitive ability and increased rate of morbidity and mortality (WHO 2006). The major cause of micronutrient malnutrition is a poor quality diet, mainly consisting of staple foods and lacking in animal products (Bouis 2003). Therefore, a balanced diet would be the best way to prevent or counteract micronutrient malnutrition, but very often people have no access to the appropriate food (WHO 2006).

In addition, the world`s population is growing rapidly and is expected to reach about 10 billion by 2050, so there will be an increased demand for food quantity and quality (Khoshgoftarmanesh et al 2010). Two current strategies exist to meet the future demands for food quantity. One is, to expand agriculturally productive farm land, which is strongly limited by several factors such as the lack of suitable land or

degradation of soils. The second is to increase the agricultural production on already existing farmland, often requiring intensive cropping practices, adaptation of high-yield genotypes and production of crops on marginal soils. This, in turn, might support the depletion of micronutrients in soil and generate crops that develop micronutrient deficiencies that intensify the problem of micronutrient malnutrition worldwide (Fageria et al 2002, Imtiaz et al 2010). The impact of environment on micronutrient concentration in the plant however strongly depends on the nutrient and also on the crop and will be discussed in chapter 1.2.2.2.

The depletion of micronutrients in soil already started in the 1960s during the green revolution, where little thought was given to nutritional value and human health. Focus was laid on food security and with it on the application of nitrogen, phosphorous and potassium fertilizers for the production of high-yield varieties (HYV), but not on mineral fertilizers. It is estimated that nowadays about 50% of the yield can be attributed to the application of nutrient fertilizers (Stewart et al 2005).

However, today many plants have low micronutrient contents in the edible part and many countries cannot meet the micronutrient demands of their people (McIntyre et al 2001). But also plants rely on an adequate supply with minerals from soil for growth and health to maintain or reach the desired quality and quantity (Borg et al 2009).

Moreover, the green revolution exclusively concentrated on the production of rice, maize and wheat to supply the poor with enough energy, often at the cost of dietary diversity and micronutrient output (Welch and Graham 1999). Along with this the prices for staple crops decreased and prices for other plants increased, making the latter unavailable for the poor (Tanumihardjo et al 2008). The introduction of green revolution food crops in South Asia increased the amount of available energy per capita, but at the same time led to a strong increase in iron deficiency anemia (Welch and Graham 1999). In developing countries undernutrition and overnutrition often occur simultaneously in different population groups even in individuals living in the same household. Data, documenting this phenomenon, also referred to as double burden of malnutrition, show increasing rates of overweight and obesity in children and adults, and slow progress in reducing undernutrition (FAO 2006, Lee et al 2010).

To solve the existing problems it is indispensable for agriculture to formulate new policies focusing on both energy and nutrient supply (IFPRI 2006).

Four main public health strategies exist to prevent and combat micronutrient deficiencies. Three of them are food based strategies (dietary diversification, fortification and biofortification), the 4th is supplementation (White and Broadley 2005). These strategies are often complemented by nutritional education programs and public health interventions in order to control parasites and infectious diseases (Stein 2010).

Dietary diversification aims at increasing access, availability and utilization of nutrient dense foods throughout the year. To reach the desired goals the implementation of this strategy requires, in a first step, the knowledge about dietary habits, such as food production, processing and food selection of the targeted population. In a second step dietary habits have to be modulated and the acceptance of these changes has to be assured (Gibson and Hotz 2001). Until today no significant, notable changes in the prevalence of micronutrient malnutrition can be attributed to dietary diversification programs (GAIN 2009). In contrast, Homestead Food Production Programs, which integrate small live-stock production and nutrition education into existing home gardening programs have been shown to be efficacious in reducing the prevalence of anemia (HKI 2010).

The traditional interventions such as supplementation and fortification have substantially reduced morbidity and mortality in developing countries. The reduction of child mortality after Vitamin A supplementation and the decreased prevalence of severe disabilities of newborns following folic acid fortification are only two out of numerous success stories (GAIN 2009). But both interventions have failed to be universally successful for various reasons (Mayer et al 2008), leaving more than 2 billion people still affected by micronutrient malnutrition, most of them living in developing countries (GAIN 2009). However, each of the traditional strategies has their particular strengths and weaknesses. Until today, fortification, adding micronutrients to food, is considered to be the most cost effective and sustainable approach to deliver minerals and vitamins to large populations (Manner and Sankar 2004). It is estimated that up to 70% of a population can be reached by iron fortification with relatively little cost and according to the Copenhagen Consensus fortification ranks third in terms of international development priorities (cost- benefit-analysis) (Horton et al 2008). Supplementation, the periodic administration of pharmacological micronutrient concentrations, is the most appropriate approach for acute cases of micronutrient deficiencies (Ortiz-Monasterio et al 2007), but less

suitable for a sustained treatment of large populations (Gomez-Galera et al 2010). One of the reasons for the only fragmentary success of some supplementation as well as fortification programs is the lack of infrastructure to reach more remote rural, traditional communities in the developing world, which are often the areas with highest prevalence of micronutrient malnutrition. Often, people with greatest need, where processed foods are largely unavailable and less affordable or the health care infrastructure is poor, are not reached (Palmer and West 2010). Both fortification and supplementation interventions often exhibit low or inadequate coverage, in the case of supplementation due to irregular supply (Lutsey et al 2008) and in the case of fortification due to the lack of centrally processed food vehicles or/and less developed commercial markets and technologies. Furthermore, a big issue is the often missing compliance in the case of fortification mainly due to higher prices of fortified foods (GAIN 2009, Qaim et al 2007, Seck and Jackson 2008), although this should not be a problem with national programs of staple food fortification. Aside from that all traditional approaches require political stability and continued investment (White and Broadley 2005) and further improvements, focusing on product stability, absorption, costs and development are necessary (Venkatesh Manner and Sankar 2004).

Biofortification of staple foods could be a more sustainable strategy, also suitable for remote regions. Biofortified crops can potentially deliver iron, zinc and vitamin A to people with limited access to commercial markets (Mayer et al 2008). The suitability of biofortification for the poor, who mainly eat staples that are not commercially processed and sold but rely on household-produced crops, is the most noteworthy advantage (Tanumihardjo et al 2008). Thus, biofortification has the potential to reduce the prevalence of micronutrient deficiencies and lower the number of people requiring interventions such as fortification and supplementation (Bouis and Welch 2010).

In contrast to dietary diversification, no behavioral changes are required from the consumers. However, the target crop has to be chosen carefully, following the dietary patterns of the consumers (Qaim et al 2007). The acceptance of the newly developed crop by the targeted population, but also by the farmers, is a major issue for biofortification to be successful. To be accepted and cultivated by the farmers, the new variety must exhibit a high yield and resistance against pathogens; in short be profitable. Characteristics of the newly developed plant such as yield, micronutrient

concentration and pathogen resistance should be stable over different environments and climatic zones. Moreover, the level of micronutrients must have the potential to significantly improve human health and ensure an adequate mineral bioavailability (Bouis and Welch 2010, Nestel et al 2006).

It has been shown that micronutrient enriched plants are more resistant to diseases (mainly root diseases), and their efficient uptake of minerals from soils might result in a higher yield since minerals are required for plant growth; this effect has particularly been observed in micronutrient depleted soil (Graham et al 1999, Graham et al 2001).

Furthermore, biofortification might be a very cost effective approach. The major investments in biofortification occur during the development of the new varieties. It is estimated that the development of a micronutrient dense cultivar might cost only about $ 10 million (Khoshgoftarmanesh et al 2010), whereas other interventions such as fortification and supplementation are more cost intensive.

Once the biofortified plants are developed and grown by the farmers, seeds can be multiplied, reproduced and shared among the poor, with few additional costs occurring to maintain the high nutrient trait over time (Bouis 2003). In contrast to other interventions, requiring larger funds on an annual basis, biofortification can provide benefits to the targeted populations over years without any noteworthy further investments.

However, the cost-effectiveness of interventions depends on their impact on human health (cost- benefit ratio) and can only be estimated for biofortification at the present time. An appropriate tool to rank interventions in terms of cost-effectiveness are the Disability-Adjusted Life Years (DALYs) saved by their implementation (Meenakshi et al 2010).

### 1.1.1 The impact pathway of biofortification

For biofortification to succeed several factors have to be considered, starting with the identification of the targeted population and ending with the improvement of the nutritional status of this population. The "impact pathway for biofortified crops" as suggested by HarvestPlus is divided into the following three stages 1) discovery 2) development and 3) dissemination of the newly developed plant variety (Figure 1).

The following chapters will be a detailed description of the three stages with a focus on the development of the improved varieties.

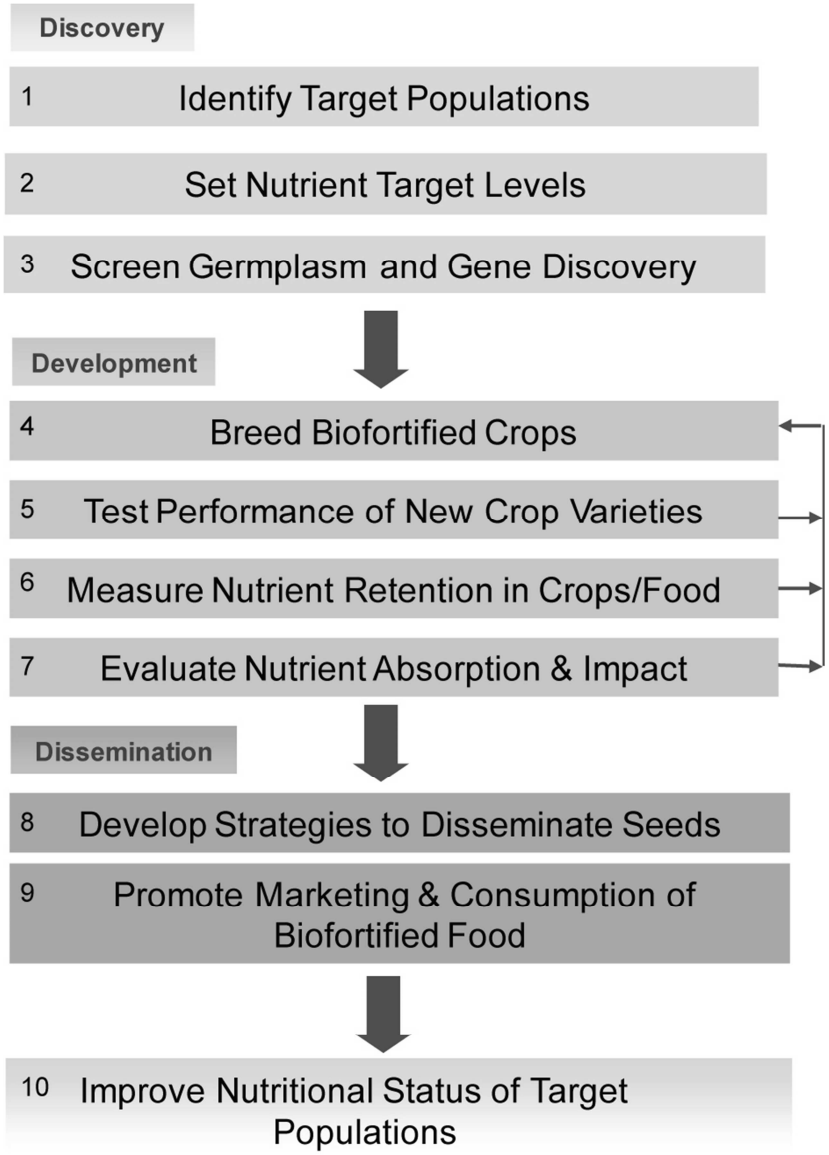


**Figure 1** Simplified diagram of the pathway for biofortified crops (HarvestPlus 2009b)

### 1.1.2 Discovery

1)

The discovery stage starts with the identification of targeted populations, for which the biofortified crop should be developed. The targeted populations are not necessarily restricted to only one country and spillover effects to other countries or

areas have to be taken into consideration. The selection should be done with regard to the prevalence of micronutrient deficiencies, the production and consumption of the targeted crop and the proportion and importance of self- or locally produced plants (Ortiz-Monasterio et al 2007). Fragmentary or missing data from national health surveys complicates the identification of populations effected by micronutrient deficiencies in many cases (Zapata-Caldas et al 2009). Further, to correctly assess the consumption of the targeted crop in a population, the availability of representative and reliable dietary intake data has to be assured (Hotz and McClafferty 2007). Information about food production might be obtained from FAO production statistics and food balance sheets (FAO 2011).

2)

The appropriate target levels for micronutrients in the biofortified crop have to be set (Table 1). The setting of target levels should be done in co-operation of plant breeders and nutritionists (Ortiz-Monasterio et al 2007).

Nutritionists estimate the micronutrient concentration, which is necessary to have an impact on the nutrition and health status of the targeted populations. For that, as mentioned above, the daily consumption of the crop has to be assessed, an exercise which has proven to be difficult since dietary intake data of many countries are often incomplete or inexistent. The retention of the nutrients following processing and cooking has to be taken into consideration as well as the bioavailability of the minerals after processing when eaten in a traditional diet (Hotz and McClafferty 2007). Bioavailability of minerals is mainly influenced by the concentration of inhibitors and enhancers in the food (Hallberg 1981), and their concentration in turn is strongly depending on food processing and dietary habits. It has been shown that soaking strongly effects the concentration of inhibitors in the food mainly due to their leaching into the water. However, if the water is not discarded but rather consumed, soaking has only minor effects (Luo et al 2009, Shimelis and Rakshit 2007). Detailed information about nutrient retention can be found in the USDA Table of Nutrient Retention Factors. The effect of different processing methods on nutrient retention is listed for 16 vitamins and 8 minerals for about 300 different foods (USDA 2007). An overview of the assumptions used to set micronutrient target levels are given in Table 1.

**Table 1** Assumptions made to set micronutrient target levels for biofortified crops (Bouis and Welch 2010)

| Amount eaten or nutrient | Criteria | Rice (polished) | Wheat (whole) | Pearl millet (whole) | Beans (whole) | Maize (whole) | Cassava (fresh wt.) | Sweet potato (fresh wt.) |
|---|---|---|---|---|---|---|---|---|
| Per capita consumption | Adult women (g/d) | 400 | 400 | 300 | 200 | 400 | 400 | 200 |
| | Children 4–6 yr (g/d) | 200 | 200 | 150 | 100 | 200 | 200 | 100 |
| Fe | % of EAR† to achieve | | | | ~30 | | | |
| | EAR, nonpregnant, nonlactating women (µg/day) | | | | 1460 | | | |
| | EAR, children 4–6 yr (µg/d) | | | | 500 | | | |
| | Micronutrient retention after processing (%) | 90 | 90 | 90 | 85 | 90 | 90 | 90 |
| | Bioavailability (%) | 10 | 5 | 5 | 5 | 5 | 10 | 10 |
| | Baseline micronutrient content (µg/g) | 2 | 30 | 47 | 50 | 30 | 4 | 6 |
| | Additional content required (µg/g) | 11 | 22 | 30 | 44 | 22 | 11 | 22 |
| | Final target content (µg/g) | 13 | 52 | 77 | 94 | 52 | 15 | 28 |
| | Final target content as dry wt. (µg/g) | 15 | 59 | 88 | 107 | 60 | 45 | 85 |
| Zn | % of EAR to achieve | | | | ~40 | | | |
| | EAR, nonpregnant, nonlactating women (µg/d) | | | | 1860 | | | |
| | EAR, children 4–6 yr of age (µg/d) | | | | 830 | | | |
| | Micronutrient retention after processing (%) | 90 | 90 | 90 | 90 | 90 | 90 | 90 |
| | Bioavailability (%) | 25 | 25 | 25 | 25 | 25 | 25 | 25 |
| | Baseline micronutrient content (µg/g) | 16 | 25 | 47 | 32 | 25 | 4 | 6 |
| | Additional content required (µg/g) | 8 | 8 | 11 | 17 | 8 | 8 | 17 |
| | Final target content (µg/g) | 24 | 33 | 58 | 49 | 33 | 12 | 23 |
| | Final target content as dry wt. (µg/g) | 28 | 38 | 66 | 56 | 38 | 34 | 70 |
| Provitamin A | % of EAR to achieve | | | | ~50 | | | |
| | EAR, nonpregnant, nonlactating women (µg/d) | | | | 500 | | | |
| | EAR, children 4–6 yr of age (µg/d) | | | | 275 | | | |
| | Micronutrient retention after processing | 50 | 50 | 50 | 50 | 50 | 50 | 50 |
| | Bioavailability ratio (µg:RE‡) | 12:1 | 12:1 | 12:1 | 12:1 | 12:1 | 12:1 | 12:1 |
| | Baseline micronutrient content (µg/g) | 0 | 0 | 0 | 0 | 0 | 1 | 2 |
| | Additional content required (µg/g) | 15 | 15 | 20 | 30 | 15 | 15 | 30 |
| | Final target content (µg/g) | 15 | 15 | 20 | 30 | 15 | 16 | 32 |
| | Final target content as dry wt. (µg/g) | 17 | 17 | 23 | 34 | 17 | 48 | 91 |

3)

At the same time, plant breeders estimate possibilities in terms of breeding additional nutrients into the plant. This includes the identification of the genetic variability of the targeted crop by screening varieties which are able to accumulate high levels of the targeted minerals (Ortiz-Monasterio et al 2007). During the screening process, lines have to be identified which accumulate and store a high proportion of the absorbed nutrients in their edible part and lines which have an increased nutrient uptake while maintaining the high proportion of nutrients in the edible part (Calderini and Ortiz-Monasterio 2003). However genetic variability is limited and bioavailability of minerals

in plant based diets often very low. Plant breeders should therefore not only focus on increasing the mineral concentration but also on increasing the mineral bioavailability from staple foods. Breeding for low/high concentrations of inhibitors/enhancers in combination with high mineral concentrations makes success of biofortification more likely (Nestel et al 2006). PA is the major cause of low mineral bioavailability from plant staples. Recently isolated low PA mutants (lpa) in wheat (Guttieri et al 2004), rice (Larson et al 2000), maize (Raboy et al 2000), barley (Larson et al 1998) and beans (Campion et al 2009) have the potential to alleviate bioavailability problems of micronutrients associated with PA. These mutants have normal phosphate levels, but reduced PA phosphate due to various mutations in the biosynthetic pathway of PA. However, the plants exhibit normal phosphate uptake and transport (Raboy 2002). So far lpa crops are in an early stage of development and most of them exhibit reduced yield and seed germination (Guttieri et al 2006, Mendoza 2002).

In addition, germplasms with greater abilities to cope with adverse climate or soil conditions should be selected. Additionally, for acceptance of the new variety by the farmers, plant breeders should focus on high yield and resistance against diseases (Ortiz-Monasterio et al 2007). The screening of different varieties is basically done in a number of international research centers as e.g. the International Center for Tropical Agriculture (CIAT) or the International Rice Research Institute (IRRI), which are in turn supported by and linked to the Consultative Group on International Agricultural Research (CGIAR).

### 1.1.3 Development

The development stage mainly focuses on the development and testing of biofortified crops. An overview of crops currently undergoing the biofortification process is given in Table 2. The identification of promising lines by breeders is followed by mapping of genotypic differences. New varieties are developed by crossing promising lines and selecting those with favorable characteristics over many generations (Grusak and Cakmak 2004). The performance of the newly developed biofortified varieties are then tested over different environments, to asses genetic and environment (G x E) interactions. It is suggested that the variability of minerals in the germplasm depends on the genotype, the environment and G x E interaction, but the impact of the various factors differs between the minerals and crops (Oury et al 2004).

Once the desired variety is developed, the consumer acceptance in terms of taste, look and cooking quality is evaluated (Khoshgoftarmanesh et al 2010). In a next step the performance of the new variety in terms of micronutrient retention is tested, followed by the investigation of micronutrient bioavailability in humans. The later will be done by tracking the nutrients in stable or radioactive isotope absorption studies (Hotz and McClafferty 2007, Kennedy et al 2003), which will be described in more detail in chapter 1.2.2.4.

If results from these preliminary tests are promising, the performance of the new variety is investigated in an efficacy trial in human subjects, which is usually implemented as a follow up study to an absorption study. Efficacy trials aim at examining whether an intervention produces the expected results under idealized conditions. This is why efficacy trials are very closely monitored, well-controlled and conducted by highly trained specialists (Hallfors et al 2006). They require a rigorous research design including a specified and standardized treatment within standardized settings (Flay et al 2005, Flay 1986). Subjects often belong to a narrowly defined, homogenous group, who should be part of the targeted population. It has to be assured that the participants accept and comply with the treatment (Glasgow et al 2003). To reduce the probability for bias, efficacy trials usually use a randomized controlled design. Participants are randomly allocated to the intervention and control group to increase the likelihood of equal distribution of unknown factors. To further avoid bias, efficacy studies should ideally be blinded trials (Flay 1986). The strict standardization of efficacy trials allows a direct attribution of observed effects to the intervention being studied (Glasgow et al 2003).

If the outcome of the efficacy trial is positive, in a next step, the impact of the new variety on human health status is evaluated in an effectiveness trial. In this type of study the beneficial effects of the crop is tested under conditions simulating reality (Gartlehner et al 2006). This is usually done among a broadly defined population which is representative for the targeted audience (Glasgow et al 2003). The food is prepared and eaten in traditional ways within the usual household environment (Khoshgoftarmanesh et al 2010). Standardization only takes places in terms of access and availability of the biofortified crop among the population. To be sure that a crop is ready for dissemination, an effectiveness trial should be implemented since the outcome might be different from the efficacy trial and hidden difficulties, such as lack of proper implementation or weak acceptance might be uncovered (Glasgow et

al 2003, Hallfors et al 2006). It is debatable whether efficacy trials prior to effectiveness studies are necessary if the latter meet the standards of efficacy trials (Flay et al 2005).

**Table 2** Crops currently undergoing biofortification process

| Crop | Targeted nutrients | Nutrient range (µg/g) | Nutrient target level (µg/g) | |
|---|---|---|---|---|
| Rice | Zinc | 13- 58[a] | 24[j] | (polished rice) |
| | Iron | 6- 24[a] | 14.5[j] | |
| Wheat | Zinc | 25- 65[b] | 33[k] | (whole wheat) |
| | Iron | 25- 56[b] | 52[k] | |
| Maize | β-Carotene | 5- 8.6[c] | 15.5[c] | (whole maize) |
| | Zinc | 13- 58[d] | 55[c] | |
| | Iron | 10- 63[d] | 60[c] | |
| Cassava | β-Carotene | 0.1-20[e] | 15.5[k] | (fresh wt.) |
| Beans | Iron | 53- 112[f] | 94[k] | (whole bean) |
| | Zinc | 20- 55[g] | 49[k] | |
| Sweet potato | β-Carotene | 0-100[h] | 32[k] | (fresh wt.) |
| Perl millet | Iron | 47 (average)[k] | 77[k] | (whole pearl millet) |
| | Zinc | 47 (average)[k] | 58[k] | |

[a] (Gregorio 2002); [b] (Ortiz-Monasterio et al 2007); [c] (HarvestPlus 2006b); [d] (Bänziger M. and J. 2000); [e] (Iglesias et al 1997); [f] (Blair et al 2010d); [g] (Beebe et al 2000); [h] (van Jaarsveld et al 2005); [j] (Hotz and McClafferty 2007); [k] (Bouis and Welch 2010)

#### 1.1.3.1 Traditional plant breeding versus genetic engineering

This chapter discusses the progress in biofortification of different crops and the targeted nutrients, directly comparing genetic engineering and traditional plant breeding techniques.

Conventional plant breeding and genetic engineering both involve changing the genotype of targeted crops with the aim of developing plants carrying genes that

support the accumulation of bioavailable minerals. The way of reaching this goal differs between the two approaches (Gomez-Galera et al 2010).

As already mentioned above, the main nutrients targeted for biofortification are beta-carotene, iron and zinc. Most work is currently done on traditional plant breeding techniques, exploiting the variability of mineral concentrations found in different germplasms (Qaim et al 2007). Not all crops have the genetic potential to meet desired micronutrient levels with traditional plant breeding, and therefore genetic engineering has to be applied to achieve sufficient improvements (Borg et al 2009). It is suggested that genetic modification is an excellent approach to obtain high micronutrient concentrations (Bouis 2007) and that genetically modified organisms (GMO) have the potential for increased agricultural productivity. A positive factor is the fast development and stable expression of GMO traits. To receive the desired new variety far fewer breeding generations are needed with genetic engineering compared to traditional plant breeding. Additionally, genetic engineering is more precise since single genes can be introduced in the targeted plants. But usually patented or patentable inventions are associated with the developed GMOs, making them inaccessible for researchers in developing countries and unaffordable for farmers (Pardey et al 2000). About 70 % of investments for the development of genetically modified plants come from the private sector, situated in developed countries, with hardly any focus on nutrition and the needs of developing populations (Fresco 2003).

Aside from numerous regulatory and political restrictions, transgenic plants often have to face social and ethical considerations causing a certain resistance to them (WHO 2005). This resistance is often further intensified by existing health concerns (Seralini et al 2007). Many genes and traits in GMOs are new and their use has not proven to be safe. The application of antibiotic resistance markers, which help recovering transformed cells, is discussed controversially in literature. It might be possible that transgenes survive human digestion and reach the colon where they are taken up by intestinal microflora, leading to a transfer of antibiotic resistance genes (Netherwood et al 2004), but the risk is negligible and transfer has been shown not to occur at a detectable frequency (Demaneche et al 2008). Furthermore, it is assumed that GMOs might exhibit certain toxicity, allergenicity and carcinogenicity. Also GMOs might affect human health indirectly through negative impacts to the environment (invasion of natural habitats, reduction of biodiversity,

horizontal gene transfer; Conner et al 2003), economic or social and ethical factors (WHO 2005).

However, the focus of biofortification initiatives is mainly on the development of new plant varieties by traditional plant breeding since the approach is regarded at present as the more appropriate strategy for developing countries.

#### 1.1.3.2 Micronutrient fertilizers

The third biofortification approach is the application of mineral fertilizers, which is complicated by several factors such as soil composition, mineral mobility in the plant and its accumulation sites (Hirschi 2009). Depending on the soil pH, most soil-applied micronutrients such as iron become unavailable for the plant immediately after fertilization since they are rapidly bound by soil particles. This process is intensified in alkaline and calcareous soils due to the higher pH, which requires a higher quantity of fertilizers to maintain a sufficient level of minerals in the plant. This, in turn, leads to problems such as soil toxicity. Better results are achieved with the application of chelates such as EDTA since they keep the minerals in solution. But these fertilizers are more expensive (Khoshgoftarmanesh et al 2010) and bear the risk of leaching since they increase mineral mobility throughout the whole soil (Alvarez et al 2001, Gonzalez et al 2007). Another method is foliar fertilization, which is more effective than soil application. But high costs, leaf damage, low uptake and wash-off by rainfall cause considerable problems (Khoshgoftarmanesh et al 2010). Therefore the application of fertilizers is not always successful. Moreover, fertilizers must be applied regularly and are costly (Hirschi 2009), both factors not compatible with the philosophy of biofortification.

#### 1.1.3.3 The targeted crops for biofortification - state of the art

For the interpretation of literature dealing with the identification of germplasms high in nutrients, it is important to distinguish between data derived from crops grown under field conditions or in the greenhouse. Welch and co-workers (2005) found 7 times higher Fe and 4 times higher Zn values in grains from the durum wheat grown under hydroponic conditions in a nutrient solution than Ortiz-Monasterio and colleagues (2007) who investigated the same cultivar grown in the field. Furthermore, especially

in case of iron, it has to assured that the germplasm is not contaminated with iron from soil or dust (Hallberg and Bjornrasmussen 1981).
This chapter discusses wheat, rice, maize, cassava and sweet potato as potential crops for biofortification. Beans will be discussed separately in chapter 1.2.2.3.

#### *1.1.3.3.1 Wheat*

More than 3000 lines have been screened for Fe and Zn with concentrations for iron ranging from 25 µg to 56 µg per g wheat and for zinc ranging from 25 µg - 65 µg/ g wheat (Ortiz-Monasterio et al 2007). Similar values for iron were reported from Oury and colleagues (2004) who screened different sources of genotypes in different environments. They observed high G x E interactions for zinc and iron and low G x E interactions for Mg and suggested that breeding for high concentrations of Fe and Zn might be difficult. Substantial impact of G x E interactions on mineral concentration was confirmed by a recently conducted study in India (Joshi et al 2010), and it is suggested that genetic factors for zinc and iron concentration in the wheat plant are of minor importance. Furthermore milling, which is accompanied by the removal of the seed coat and embryo, leads to a substantial decline in iron concentration (40 %; Borg et al 2009). However, iron and zinc correlate positively in wheat (Zhang et al 2010) and highest concentrations (up to 85 µg /g) were detected in landraces as well as in wild and primitive relatives (Ortiz-Monasterio et al 2007, Peleg et al 2008). It is proposed that crossing wild wheat relatives with high yield cultivars might be the best strategy to increase the micronutrient concentration in wheat (Calderini and Ortiz-Monasterio 2003).
The strategy of HarvestPlus is to obtain a new wheat variety by crossing high micronutrient wheat varieties with modern wheat (short stems and husk free). The newly developed wheat plant is expected to contain 40- 50 % more iron and zinc than currently cultivated varieties (HarvestPlus 2006a). However, depending on wheat intake targeted levels might be lower than the levels recommended by WHO for wheat flour fortification. To improve iron status flour fortification levels should deliver about 6 mg additional iron in the form of ferrous sulfate. Biofortified wheat varieties would deliver only about 1 mg additional iron per 100 g wheat flour (40 % losses due to milling).

Genetic engineering has been used to increase the bioavailability of minerals from some wheat varieties by expressing phytase, the enzyme for the degradation of PA, from *Aspergillus fumigatus* (Brinch-Pedersen et al 2006). However it has to be taken into consideration that the activity of enzymes is affected by heat and that the phytase will be inactivated during baking. Only little other work has been done on transgenic wheat and its transformation proved to be difficult (Shewry and Jones 2005).

#### *1.1.3.3.2 Rice*

The natural variation of iron in rice is quite low and milling and polishing usually results in a loss of up to 80 % since iron is mainly stored in the aleurone layer and not in the endosperm (Brinch-Pedersen et al 2007). Iron and zinc concentrations in rice of different genotypes (n= 1138) were found to range between 6.3- 24.4 µg/ g and 13.5- 58.4 µg/ g, respectively, suggesting that there is at least some genetic potential to successfully breed high mineral rice (Gregorio 2002). Although Fe and Zn concentrations in rice are affected by G x E interactions, high levels can be more or less maintained over different environments (Graham et al 1999).

The first study investigating the effectiveness of a biofortified crop on micronutrient status of humans was done with biofortified rice, which was produced by traditional plant breeding. The influence of a high iron (9.8 µg/ g), high yield rice on iron status of Filipino women was tested in 2005 (Haas et al). No significant increase in body iron, serum ferritin and hemoglobin was detected compared to the control group, after 9 month of feeding. Differences were only found in the non-anemic subgroup. An important limitation of the study was that the biofortified rice only provided an additional amount of 2.8 µg iron per g rice, mainly due to losses during processing, and fortification was therefore far below the desired level. This finally led to a low iron intake from rice, which only accounted for less than 20 % of total iron consumption, although the high iron rice used in the study almost contained the iron level recommended by HarvestPlus (14.5 µg /g) (Hotz and McClafferty 2007). To observe a meaningful impact of biofortified high iron rice the HarvestPlus recommendation might have to be revised, taking into account losses of up to 80 %.

Genetically modified rice, expressing soybean or *Phaseolus vulgaris* ferritin, showed an up to 2 fold increase in iron concentration in the grains (Lucca et al 2001,

Vasconcelos et al 2003), with accumulation also in the endosperm. However, the overexpression of soybean ferritin in rice did not lead to a further iron increase in the seed, only to an exhaustion of iron reserves in the leaves (Qu et al 2005). These results suggest that further iron enrichment is only feasible by increasing iron uptake or iron transport from the roots (Brinch-Pedersen et al 2007). To concomitantly increase iron bioavailability, a phytase from *Aspergillus fumigatus* was introduced into rice grains, resulting in a 130-fold higher phytase activity than in non-transformed rice grains. Unfortunately, most phytases possess only limited thermotolerance and strongly loose activity during boiling, cooking and baking (Lucca et al 2001). To further increase mineral bioavailability from rice, a gene encoding for myo-inositol synthase was suppressed, reducing PA concentration by 68 % without any effects on seed weight, germination and plant growth (Kuwano et al 2009). Other workers introduced a nicotineamine synthase gene in rice grains to increase mineral concentration. Nicotineamine is a metal chelator which is also involved in metal assimilation and homeostasis and might be one of the limiting factors of mineral accumulation. Plants with higher concentrations in nicotineamine were found to have higher amounts of Fe and Zn in leaves and seeds (Lee et al 2009). Wirth and colleagues (2009) developed a transgenic high nicotineamine rice and observed 6-fold higher iron concentrations in the endosperm, when compared to the wild type.

Much research has been done on β- carotene enrichment of rice grains for over a decade. Rice, which normally contains beta-carotene only in the leaves, was genetically modified to produce carotenoids in the endosperm. Several generations of the so called "Golden Rice" have been developed until today, the latest containing up to 37 µg carotenoids per g rice (Paine et al 2005). A recently conducted study in humans showed that Golden rice β- carotene is effectively converted into retinol with a factor of about 4:1(Tang et al 2009), which is lower compared to the conversion factor of β- carotene from vegetables and fruits (Carrillo-Lopez et al 2010)

The first Golden Rice varieties are planned to be released in the Philippines and Bangladesh in 2013 and 2015, respectively (IRRI 2011).

#### *1.1.3.3.3 Maize*

Maize is one of the major targeted plants of genetic engineering and accounts together with cotton and soybean for 85% of globally planted transgenic varieties, the

focus being on the development of insect resistant and herbicide tolerant maize (Fresco 2003). In terms of biofortification a special effort was put into breeding maize with high concentrations of provitamin A carotenoids (HarvestPlus 2006b). In the past, little focus was on high mineral maize mainly due to its low mineral concentrations and lack of genetic variability (Grusak and Cakmak 2004).

Researchers screened more than 1800 maize genotypes including improved germplasms and landraces grown in different locations and years. Iron and zinc concentrations ranged between 10 µg and 63 µg/ g and between 13 µg and 58 µg/ g, respectively, with the highest concentrations found in landraces. To investigate the environmental impact on mineral concentrations, the same germplasms were grown in six different locations. Average iron and zinc concentrations varied by 40 % and 24 %, respectively. According to the authors, environmental factors play a more significant role in terms of mineral concentration than genetic variation (Bänziger 2000). Another study investigated mineral concentrations in different maize varieties and the impact of environment, genetics and G x E interaction on mineral content. The genetic component accounted for 12 % of variability, no impact of environment was detected and G x E interactions were highly significant (Oikeh et al 2003). But results are inconsistent, and a recently conducted study found only small G x E interactions, indicating that environment has little impact (Simic et al 2009). These workers concluded that there is enough genetic variability to increase iron and zinc concentration in maize by biofortification. However, HarvestPlus researchers discovered germplasms with iron and zinc concentrations of up to 45 µg/ g and 62 µg/ g, respectively. HarvestPlus states that 60 µg/ g of iron and 55 µg/ g of zinc is sufficient to observe a significant impact on the mineral status of targeted populations (HarvestPlus 2006b).

Several studies looked at the impact of lpa maize on mineral absorption. No effect was detected on zinc absorption in Guatemalan children using maize with 60 % less phytate (Mazariegos et al 2006). Iron absorption from tortilla containing lpa maize measured by radio iron isotope techniques nearly doubled (Mendoza et al 1998), and also calcium absorption was shown to be effected (Hambidge et al 2005). To develop a maize variety with reduced PA concentration, scientists silenced the expression of a transporter in normal maize, whose defect is responsible for the low PA content in lpa mutants. The newly developed maize had a reduced PA concentration, but in contrast to lpa, showed no cutback in germination and seed dry weight (Shi et al

2007). Some work has also been done on the expression of recombinant soybean ferritin and *Aspergillus* phytase in maize to increase the level and bioavailability of iron. Iron concentration increased significantly and phytate was found to be almost totally degraded in flour paste produced from the genetically modified maize (Drakakaki et al 2005).

#### *1.1.3.3.4 Cassava*

Cassava, an important crop in many developing countries, contains iron and zinc only in low concentrations. Thus, the focus of biofortification initiatives is exclusively on increasing beta- carotene concentration (Montagnac et al 2009). The variation of carotenes in cassava is high and strongly depends on the root color. White varieties have by far the lowest concentration (1.3 µg/ g), whereas highest concentrations can be found in orange varieties (12.6 µg/ g). Iglesias and co-workers (1997) analyzed 632 accessions from the CIAT germplasm collection of 5500 accessions. They detected germplasms with beta- carotene concentrations above 20 µg/ g, suggesting a high genetic variability that would make it possible to successfully biofortify cassava and meet the daily retinol requirements of adults. However, it also has to be taken into consideration that carotene concentration is effected by thermal processing.

#### *1.1.3.3.5 Sweet potato*

The major aim of the biofortification programs is the replacement of white fleshed low provitamin A sweet potato varieties with orange fleshed high provitamin A plants. HarvestPlus has set the target level for sweet potatoes at 32 µg/ g (HarvestPlus 2009e), but varieties with concentrations up to 100 µg/ g already exist (Nestel et al 2006). A study conducted in Durban, South Africa observed a significant improvement in vitamin A status of children, when fed with orange fleshed sweet potatoes. Workers provided the children with either orange fleshed potato with a beta carotene concentration of about 100 µg/ g in the cooked root or white fleshed potato without any beta- carotene over a period of 11 weeks (van Jaarsveld et al 2005). Vitamin A liver stores were significantly increased in the treatment group compared to the control group. Furthermore it has been shown that the retention of beta- carotene

from orange fleshed sweet potatoes when boiled is very high with about 80 % of the initial concentration (van Jaarsveld et al 2006).

#### 1.1.3.4 Phaseolus vulgaris- A vehicle for iron biofortification?

The domestication of *Phaseolus vulgaris* (common bean) occurred independently in South America and Central America/ Mexico, leading to two different domesticated gene pools; the Andean and Mesoamerican, respectively (Debouck et al 1993). The Mesoamerican gene pool is compared to the Andean gene pool (one phaseolin type, "S") characterized by larger seeds and two phaseolin types ("T" and "C"). Phaseolin is the major seed storage protein in beans and its analysis revealed three predominant banding patterns (S= Sanilac, T= Tendergreen, C= Contender) giving reliable information about the corresponding gene pools (Gepts and Bliss 1986, Gepts et al 1986). The Mesoamerican gene pool is represented by pinto, pink, black, white and some snap beans, whereas the Andean is represented by kidney, cranberry and many snap beans (Talukder et al 2010).

The common bean is estimated to be the major staple food for over 300 million people in Eastern Africa, Meso- and South America and for this reason is one of the most important legumes worldwide (HarvestPlus 2009a). The annual production is about 8.5 million metric tons in the developing world (Broughton et al 2003). Most of it is produced for own usage on small farms ranging from 1 to 10 ha, with 4 million hectares planted annually. African countries with the highest production are the Democratic Republic of the Congo (DRC), Kenya, Tanzania and Uganda, whereas the highest per capita consumption is found in Burundi and Rwanda (Blair et al 2010a). The per capita consumption in Rwanda and Burundi is estimated to be about 66 kg and 31 kg per year, respectively (Broughton et al 2003). Ninety- five percent of Rwandese and Burundian farmers cultivate beans, accounting for about 30 % of cultivated land, with a productivity of 800- 1000 kg/ha (Blair et al 2009, Blair et al 2010a). The bimodal rainfall in Eastern Africa normally enables bean planting twice a year, leading to a fairly high production. Common beans are self-pollinating, diploid plants, which also can be differentiated with respect to their growing habits, ranging from determinate or indeterminate bush beans to climbing beans (Blair et al 2010c). Although favored by the farmers and being the predominant species, bush beans are more and more replaced by new varieties of climbing beans. These new bean

varieties have already been released in Eastern and Central Africa (Rwanda, Burundi, DRC, and Kenya). They exhibit a higher yield in small space, large grains, good nitrogen fixation, reduced vulnerability to certain diseases and flexibility for various cropping systems (e.g. maize x beans), making them a suitable crop for small farms (Blair 2009, CIAT 2010). Bush beans produce a crop in about 65 days and yield below 600 kg/ ha per season in Eastern and South Africa. The growing season of climbing beans, on the other hand, ranges between 100- 120 days, with yields up to 4.5t/ ha per season. Usually, the advantages of bush beans are the two growing seasons per year, but some, early maturating and low altitude adopted climbing beans for twice yearly planting are available (Blair 2009). The cultivation of beans faces biotic (e.g. fungal and viral diseases) as well as abiotic constraints (acid soils, low soil phosphorus), and their cultivation on steep hillsides increases the risk of erosion. However, insect and disease control by chemicals is quite uncommon, but biocontrol methods and crop rotation have been recommended (Allen et al 1989). Phenotypic variety, especially in Rwanda is high, although urban consumers demand more and more pure lines instead of mixtures, and traditional varieties are replaced by new ones (Blair et al 2010a). In Eastern Africa the common bean belongs to the most crucial sources of proteins (65 % of consumed protein) and energy (32 % of consumed energy; Blair et al 2010a, Broughton et al 2003, Welch et al 2000), and provides people with iron and zinc, as well as vitamins such as thiamin and folic acid (Broughton et al 2003, Pennington and Young 1990, Souci et al 1994).

#### 1.1.3.5 Genetic variation of iron in common bean seeds

More than 36000 accessions of beans for 44 species of *Phaseolus* from 109 countries are held in a gene bank at CIAT, rendering it the most diverse and largest bean collection worldwide (CIAT 2011). For iron uptake from the soil, plants have developed two different strategies. In low iron conditions some plants activate a reduction based strategy (strategy 1), and others such as corn and wheat use a chelation-based strategy (strategy 2). Beans belong to strategy 1 plants. To make iron available in the soil it is reduced by rhizosphere acidification and iron reductases (Frossard et al 2000).

It has been observed that Andean and Mesoamerican genotypes differ in seed mineral concentration, with Andean beans having higher iron concentrations and

Mesoamerican being higher in zinc (Islam et al 2002). The same study revealed a higher activity of iron reductase in Andean beans. Furthermore, it has been shown that seed iron accumulation correlates with reductase activity, indicating a relation between root uptake and seed loading of iron (Blair et al 2010b). Recent studies identified the quantitative gene loci (QTL) controlling iron and zinc accumulation (Blair et al 2009, Blair et al 2010c). These findings are promising for the use in biofortification, since the marker assisted selection of high micronutrient beans might speed up the breeding process and save plant material.

One hundred nineteen wild and 1031 cultivated genotypes were screened at CIAT and it has been shown that there is a promising genetic variability for minerals in beans. The screened beans did not exhibit a correlation between geographic distribution and iron concentration, but beans from the Andean gene pool tended to have higher iron concentrations. Surprisingly, iron concentration in the wild varieties was only slightly higher than in the cultivated beans (average Fe concentration 60 µg/ g and 55 µg/ g, respectively). Iron and zinc contents of beans ranged from 35 µg - 102 µg/ g and from 20 µg - 55 µg/ g beans, respectively (Beebe et al 2000, White and Broadley 2005). Other workers reported much higher iron concentrations in wild types ranging from 71 µg/ g - 280 µg/ g (Guzman-Maldonado et al 2000), suggesting the use of these varieties in breeding programs to increase iron concentration in cultivated varieties. In general, iron and zinc concentrations were found to strongly correlate with each other (Beebe et al 2000).

Both minerals are present in higher concentrations than in cereal staples and are usually almost completely retained through harvest and processing (Beebe et al 2000, Blair et al 2010c). Although seed mineral concentrations are affected by environmental factors, the effects of genetic components show a certain stability over different environments, so that breeding for high iron and zinc should be successful (Beebe et al 2000, Blair et al 2009). An initial goal of biofortification has been the production of varieties with 80 % more iron and 40 % more zinc with selective plant breeding strategies. However, the targeted bean iron level recommended by HarvestPlus to meet a large proportion of the daily iron intake (94 µg/ g) is already in reach. Blair and co-workers (2010d) developed two promising new germplasm lines with improved nutritional quality by crossing a commercially available cultivar with a high mineral type. Their performance was tested in the Andean region and Central America. Mineral concentrations of the newly developed Andean bush beans ranged

from 53 µg - 112 µg/ g for iron and 25 µg - 43 µg/ g for zinc, depending on the planting site. Much research is currently underway to improve mineral concentration not only of Andean bush beans but also of climbing beans and bush beans of the Mesoamerican gene pool.

Another approach focuses on interspecific crosses with Phaseolus coccineus, which exhibits iron concentrations up to 127 µg/ g (Blair 2009).

However, the work currently done clearly reveals that genetics as well as the environment play an important role in terms of mineral concentration. Although high iron and high zinc genotypes will accumulate more of these nutrients than low iron and low zinc germplasms grown at the same location during the same growing season, the major challenge will be to maintain sufficient mineral concentration over varying climate, altitude and soils types. Furthermore, it has to be considered that iron bioavailability from beans is reported to vary from 1 % to 3 %, due to the high concentration of PA and PP (Beiseigel et al 2007, Donangelo et al 2003, Lynch et al 1984). While PP concentration strongly varies with the bean color and variety, PA concentration is constantly high (Anton et al 2008, Beebe et al 2000, Towo et al 2003) and increases with iron concentration (Hoppler et al unpublished). The speciation of iron in beans has not been extensively studied, but it is suggested that ferritin bound iron accounts for 15- 30 % of total iron (Hoppler et al 2009). Recent results from our laboratory (Hoppler et al unpublished) with beans of varying iron content indicate that the concentration of ferritin bound iron is similar in all beans and that bean iron is increased by an increase in non-ferritin iron, possibly linked to phytic acid.

The development and use of lpa beans might be a step towards overcoming low iron bioavailability. Campion and co-workers (2009) isolated a lpa bean mutant by chemical mutagenesis. This bean exhibited low PA concentration without any defects in terms of growth and yield. However, low PA concentrations might not have a positive effect on iron bioavailability due to the presence of PP since it has been shown that removing phytate in the presence of high PP concentrations did not affect iron bioavailability from beans.

It is assumed that in the near future, the target levels (see Table 2) for zinc will be achieved for rice, wheat maize and pearl millet and iron levels will probably be reached in beans. For crops not possessing the genetic potential for the desired

accumulation of nutrients, as in the case of rice and provitamin A, HarvestPlus considers genetic engineering as a possible approach (Hotz and McClafferty 2007).

#### 1.1.3.6 Isotope studies- A tool to measure the impact of iron biofortification

Iron deficiency (ID) is the most prevalent micronutrient deficiency worldwide, affecting mainly children under five and women of child bearing age living in the poorer communities of the developing world (McLean et al 2008). ID has a major negative impact on health and contributes to the risk of severe anemia, which is associated with impaired physical development and higher maternal morbidity and mortality (WHO 2007).

Stable and radio iron isotope absorption studies are a widely used tool to measure iron bioavailability from foods. To measure iron absorption, an extrinsic tag is usually added to one or several test meals. Venous blood samples are drawn 14 days after test meal administration and samples are analyzed for iron isotope incorporation in the erythrocytes. The calculation of absorption requires the estimation of blood volume and of the fraction of absorbed iron incorporated into red blood cells (80 %; Brown and Hopper 1962, Roughhead and Hunt 2000).

The use of extrinsic tags has been shown to deliver comparable results to intrinsic isotopic labeling, which is the introduction of mineral isotopes in the plant via stem injection or hydroponic culture. The comparable results between intrinsic and extrinsic tagging are due to the fact that non heme iron from foods enters a common, exchangeable iron pool (Björn-Rasmussen et al 1973, Cook et al 1972, Donangelo et al 2003, Grusak 1997).

However, to successfully tag foods, a complete exchange between the native non-heme iron and the extrinsic tag is necessary. This can be achieved by mixing the tracer well with the food and if necessary milling of the test meal components. Workers detected a difference in the absorption of extrinsically and intrinsically labeled iron from unmilled unpolished rice. It was proposed that the outer layer of the rice grain prevented the iron exchange since the rice grain does not break up in the stomach. Repeating the experiment with milled and polished rice grains led to the expected results (Björn-Rasmussen et al 1973).

To gain preliminary information about the bioavailability of iron from iron biofortified crops, stable or radio iron studies can be used as pilot studies to efficacy trails. The

results of absorption studies assist plant breeders as well as nutritionists in the determination of nutrient target levels. But they also simplify the planning of efficacy trials in terms of sample size calculation and the frequency as well as the size of meals administered during the study.

#### *1.1.3.6.1 Single vs. multiple meal studies*

Two different designs of iron isotope absorption studies can be used; single and multiple meal studies. In single meal studies, one single test meal containing the total iron isotope is administered on a single day and absorption is measured. Multiple meal studies involve the administration of several meals all containing a small amount of the isotope over a longer period. Most information about iron bioavailability from foods is based on results of iron absorption from single meals and little information is available about iron absorption from complex diets fed over a longer period (Cook et al 1991a). Several workers have observed differences between iron absorption from single meals and multiple meals (Cook et al 1991a, Tidehag et al 1996).

Tidehag and co-workers (1996) measured iron absorption from standardized meals that differed in the amount of iron, provided 3 times a day over a period of 5 days. They labeled the breakfast meals of day 4 and 5 differently from the other meals and compared absorption. Absorptions from the breakfast meals were 50-80 % higher than from multiple meals. The scientists proposed that the observed difference was either due to a lower iron intake from the breakfast meals in the morning, leading to higher absorption or due to the 12 h overnight fast before breakfast, compared to 4 h without food before lunch and dinner.

Cook and colleagues (1991a) measured iron absorption from different single meals (moderate, low and high bioavailability) and compared those results with absorption from diets (moderate, low and high bioavailability) fed over two weeks. Subjects were in most instances free to choose their diets, but the high bioavailability diet had to contain a minimum amount of meat and vitamin C. Additionally, subjects were not allowed to drink coffee or tea or to eat bran and eggs. Subjects consuming the low bioavailability diet were not allowed to consume meat and vitamin C containing foods, but were requested to eat legumes, cereals, and foods rich in bran. The workers observed a much stronger influence of enhancers and inhibitors on iron

absorption from single meals than from the complete diet, whereas absorption from single meals and complete diets with moderate bioavailability were comparable. Iron absorption from the single meal containing enhancers was nearly 6 times higher than the absorption from the inhibitory single meal, whereas iron absorption from the enhancing and inhibitory diet only showed a 2.5-fold difference. These results indicated that single meal studies overestimate the effects of enhancers and inhibitors, when compared to whole diets. The authors argued that the observed effect might have been due to a variation in iron consumption between single meal and diet since the diet was not strictly controlled. Furthermore, when compared with fasting subjects, the food residues in the stomach and duodenum of the subjects consuming the diet might have reduced the impact of enhancer and inhibitors. The authors further suggested that the observed effect might have been due to a dilution of inhibitors in the complex diet by components not affecting iron absorption.

Ascorbic acid has shown to strongly influence iron absorption in single meal studies, but multiple meal studies failed to show an effect (Cook and Reddy 2001) and large surveys failed to show a correlation between iron status and the daily consumption of vitamin C (Cook et al 1984, Hunt et al 1994). However, there is some evidence that iron status is a more important factor determining bioavailability than inhibitors and enhancers (Cook et al 1991a). Reddy and colleagues (2000) showed that serum ferritin (as a proxy for iron status) accounted for 32 % of the variability in iron absorption and that dietary factors accounted only for 16 %. The remaining 50 % of variability in iron absorption could not be attributed to iron status and dietary factors, and the workers concluded that the variability must be due to some unknown physiological factors.

It is assumed that the gap in iron absorption between enhancing and inhibiting meals gets smaller over the long term and the level of absorption depends on the iron status of the participant. These iron status related adaptation processes were investigated by Hunt and Roughead (2000). They conducted a study looking at iron absorption from meals with high and low bioavailability over a 10 week period in men with normal iron status. Absorption was measured at the beginning and at the end of the study. Adaptation reduced the difference between the low and high bioavailability diet from 8- to 4- fold over time, despite a reduction in serum ferritin of the subjects.

The adaptation of iron absorption to maintain body iron stores cannot explain the results of a study conducted in iron deficient women (Hunt et al 1994). Participants of

that study were supplemented with vitamin C, given with the meals. No changes in serum ferritin were observed. These results give rise to another theory, suggesting that the adaptation of iron absorption might be caused by an adjustment of the human body to the presence of inhibitors and enhancers, independent of iron status (Hunt 2003).

However, it is in general questionable if a measureable adaptation already takes place within the first days of consumption and with it explains the conflicting results observed in single and multiple meal studies.

It can be stated that multiple meal studies are an appropriate tool to determine iron bioavailability from whole diets, especially in the presence of enhancers and inhibitors and that single meal studies might lead to biased results. As the iron status and inflammatory status of the subject influence iron absorption, multiple meal studies give a useful approximation of absorption from diets of high and low iron bioavailability. However, all enhancers and inhibitors have been identified with single meal studies, clearly showing that they are a suitable tool for the identification of factors influencing iron bioavailability. Furthermore they are less time consuming and more cost effective compared to multiple meal studies. To sum it up, multiple meal studies give more accurate results than single meal studies in specific situations, but are not always needed.

### 1.1.4 Dissemination

The dissemination stage starts with the development of a strategy on how to multiply biofortified planting material and create markets for the biofortified seed and crops (Bouis and Welch 2010, HarvestPlus 2009b). This does not only include the provision of biofortified seeds and the promotion of the biofortified crops among farmers but also the achievement of behavioral changes at policy, institutional, community and individual levels (HarvestPlus 2009d). To successfully disseminate new cultivars, an integrated approach involving all stakeholders is indispensable. This requires a great deal of coordination as well as networking and facilitation of interactions (Erenstein 2003). The implementation strategy, according to HarvestPlus starts with the analysis of existing data on the production- marketing- consumption chain of the targeted country (HarvestPlus 2009c). Subsequently, the focus will be on the adoption of the new variety by farmers and the production and dissemination of seeds through

traditional and non-traditional channels. Marketing strategies will be developed for promoting the biofortified variety, and finally a demand for the new product has to be created. This whole process is to be constantly monitored and necessary modifications will have to be made. However, many challenges might be faced, and it is reported that the major bottleneck for the success of a newly developed variety is seed multiplication and distribution.

In a first attempt, the dissemination of improved bean varieties in Malawi failed mainly due to insufficient efforts of multiplication, promotion and distribution (Chirwa et al 2006). To eventually succeed, a new strategy was developed including seed multiplication by contract growers (trained farmers) and secondary seed multipliers. These secondary seed producers were reached through NGOs or government initiatives, which were supplied with seeds for distribution. To further stimulate demand several promotion channels such as print media and mass media were used. Distribution of the newly developed varieties occurred via research stations, government extension agents, NGOs, rural grocery shops, health clinics, primary schools and village centers. The authors of this study concluded that for any seed program to be sustainable and successful in the long term it must be profitable in order to attract private traders.

While the implementation of a new bean in Malawi was successful, it failed in Tanzania. According to the researchers, the major reason for this failure was the inaccessibility of seeds, caused by insufficiently elaborated dissemination strategy. This in turn strongly contributed to a low adoption of the new variety (David et al 2002).

It can be summarized that biofortification is still a rapidly developing field which has to face considerable challenges. To date, many promising high mineral germplasms have been identified, showing that there is enough genetic variability in most staple crops to breed for high micronutrient concentrations. However research also revealed that the often strong influence of environment on mineral concentration complicates the breeding process. Until today, it has not been possible to develop a new plant variety maintaining a high iron concentration over several seasons, soils and climate zones. High losses due to processing and low mineral bioavailability due to high concentrations of inhibitors further complicates reaching a level of iron which has the potential to positively influence human health. This indicates that, at least for staple

crops with insufficient genetic variability or other limiting factors, genetic engineering should be taken into consideration as a future strategy.

## 2 Iron absorption inhibitors and enhancers

The absorption of non-heme iron, which makes up over 90 % of the iron in an average diet, varies strongly mainly influenced by the concentration of enhancers and inhibitors and the iron status (Hunt 2002).

*Enhancers*

The main enhancer of iron absorption from plant foods is ascorbic acid. It increases iron absorption by forming soluble complexes with iron and/or reducing iron to its soluble ferrous form (Hurrell 1997, Lynch and Cook 1980). Although it has the ability to overcome the inhibitory effect of phytate, no ascorbic acid is naturally present in cereal grains and legume seeds. Furthermore ascorbic acid is highly sensitive to oxygen and heat and will be degraded to a large extent during storage, processing and cooking procedures prior to consumption (Hallberg et al 1989). Other organic acids, such as lactic acid (Derman et al 1980) or citric acid (Derman et al 1987), seem to have the ability to positively influence iron absorption, probably by chelating iron and increasing its solubility. Results are less clear and, in parts, contradictory (Hallberg and Rossander 1984). To observe a positive effect on iron absorption, high concentrations of organic acids are necessary, which in turn, might lead to undesirable organoleptic changes in most foods (Teucher et al 2004).

The enhancing effect of meat on non-heme iron absorption is ascribed to the so called "meat factor". Its existence is well accepted and has been demonstrated in several *in vivo* studies (Bjornrasmussen and Hallberg 1979, Boech et al 2003, Hallberg and Rossander 1984), but until today the identification of its structure and mechanism of action failed. One proposal is that the "meat factor" consists of iron binding peptides, which protect iron from the complex formation with inhibitors and thus, keep it in solution (Storcksdieck et al 2007).

*Inhibitors*

The two major iron absorption inhibitors are PA and PP. Other compounds that negatively influence non-heme iron absorption are numerous and among them are proteins from plants and animals. Egg white proteins (egg albumin; Monsen and Cook 1979) and milk proteins (casein; Hurrell et al 1989) were reported to inhibit iron absorption, but to a lesser extent than soy protein isolates (Hurrell et al 1992).

Calcium is a further candidate suspected to impair iron bioavailability, but results are conflicting and inconsistent. In order to significantly reduce iron absorption calcium has to be present in high concentrations (Cook et al 1991b, Monsen and Cook 1976). However, Troesch and colleagues (2009) found no impact of calcium on iron absorption, even though they added 200 mg to a test meal. A dose dependent effect of calcium on iron absorption has been shown by Hallberg and co-workers (1991). In that study, 40 mg of calcium did not influence iron absorption, but 75 mg already reduced iron absorption by about 16 %.

The following review concentrates on PP and inulin/oligofructose, the latter potentially increasing iron absorption. A short overview will be given on the occurrence of PA and its influence on mineral absorption. All three compounds are present in varying amounts in plant foods, such as legumes and wheat, and consequently are part of the diet consumed in developing countries. Breeding plants with low PP and PA concentrations as well as with high inulin concentrations might be an approach to improve iron status in these countries and thus might play an important role in formulating breeding strategies.

## 2.1 Polyphenols

### 2.1.1 Nomenclature

Polyphenols are a heterogeneous class of compounds derived from the secondary plant metabolism. Secondary metabolites are components without any function in growth, photosynthesis or reproduction of the plant. Nevertheless PP have important functions in the plant. They serve the plant as protection against pathogens, with the polymeric compounds that are more toxic to phytopathogens than monomers. They also protect the plant against UV radiation (Friedman 1997, Manach et al 2004) and their ability to absorb light in the visible range makes them important for pollination by insects (Sisa et al 2010).

All PP found in non-plant material, such as animal tissue, are of plant origin and appear there as a result of the consumption of plant foods. Chemically, phenolics can be defined as compounds with an aromatic ring bearing at least one hydroxyl moiety

(Shahidi and Naczk 1995). They are usually divided into the following groups (Figure 2): (1) lignans and polymeric lignins (2) stilbenes (3) phenolic acids with the subgroups benzoic acids and hydroxycinnamic acids and (4) flavonoids (Han et al 2007).

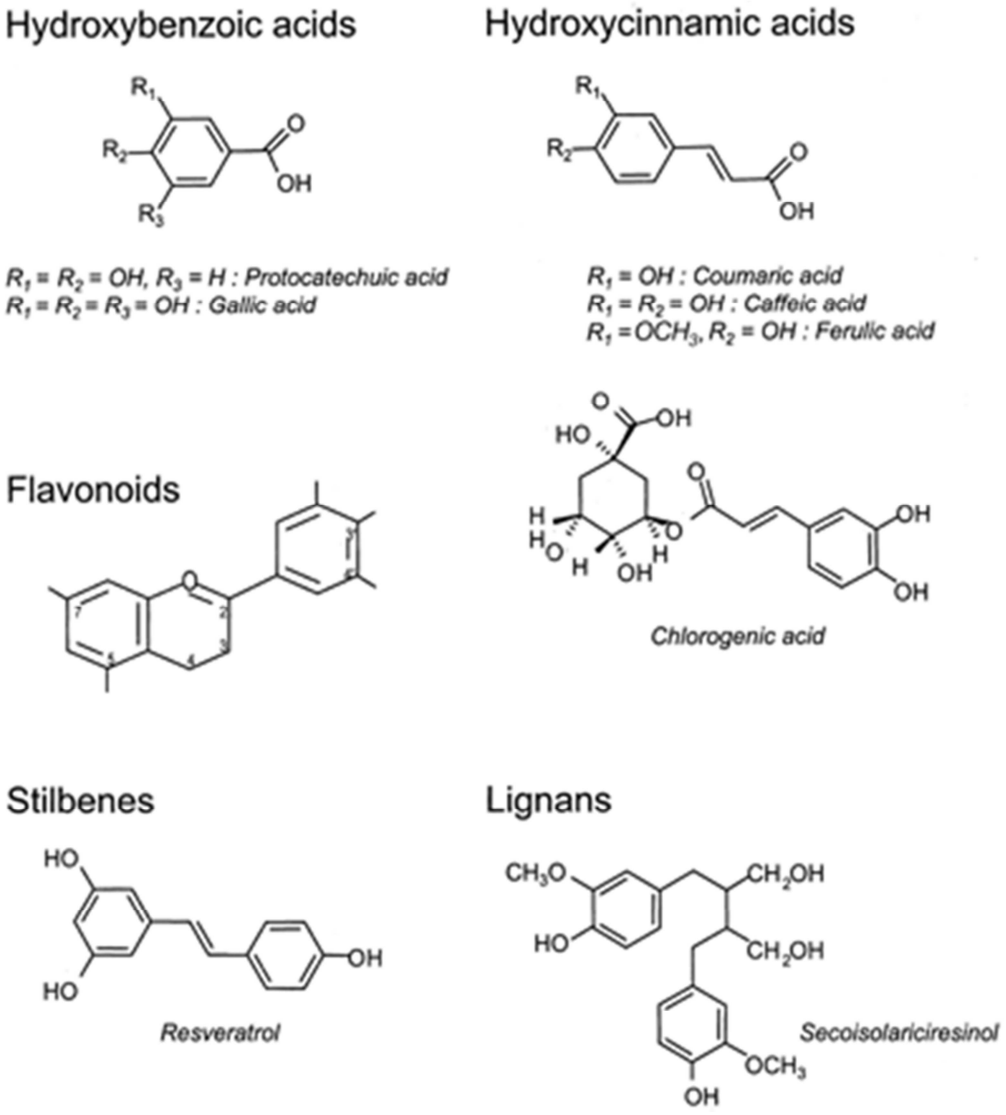


**Figure 2** Chemical structures of polyphenols (Manach et al 2004)

#### 2.1.1.1 Lignans and stilbenes

Polymeric and insoluble lignans exhibit certain estrogenic characteristics and are the most ubiquitous phenolic compounds and are found in all higher plants with lignified tissues plants (Shahidi and Naczk 1995). Stilbenes consist of two aromatic rings

linked by an ethane bridge, and can be present in different forms (monomers-polymers, glycosides). The most prominent stilbene is trans-resveratrol, which is present in many foods and beverages, but most prominently in grapes and wine (Fulda 2010, Huang et al 2010).

#### 2.1.1.2 Hydroxybenzoic acids

Hydroxybenzoic acids are usually esterified with a polyol moiety, in most cases a *D*-glucose. Considerable amounts of free hydroxybenzoic acid derivatives such as gallic acid or 4-hydroxybenzoic acid, are found in red fruits, spices and herbs, and green and black tea (Shahidi and Naczk 1995, Tomas-Barberan and Clifford 2000). Hydroxybenzoic acids are normally present in food, bound to complex structures like hydrolysable tannins or lignins. Hydrolizable tannins are either composed of esterified gallic acid units (gallotannin) or esterified hexahydroxydiphenic acid units (ellagitannins; Haslam 2007).

These complex hydroxybenzoic acids are present in plant families such as *Leguminosae* and *Rosaceae* (e.g. beans, peas, lentils, nuts, apple, pear, raspberry, plum and cherry), and occur often associated with proanthocyanidins (also condensed tannins; Shahidi and Naczk 1995) or, as in the case of gallic acid, as part of the proanthocyanidin structure (Haslam 2007, Serrano et al 2009).

#### 2.1.1.3 Hydroxycinnamic acids

Derivatives of hydroxycinnamic acids are more common than hydroxybenzoic acids and are primarily represented by p-coumaric, caffeic, ferulic and sinapic acids, but they rarely occur in their free form, except in processed food. The most abundant phenolic acid, both free and esterified, is caffeic acid since it is an intermediate of the synthesis of lignin (Manach et al 2004); although in cereal grains ferulic acid is the most common phenolic acid (Sosulski et al 1982). The best known conjugate is 5-caffeoylquinic, also called chlorogenic acid, an ester between caffeic and quinic acid. However, the term chlorogenic acid is also used for esters between quinic acid and other trans-cinnamic acids (Clifford 2000).

### 2.1.1.4 Flavonoids

More than 8000 flavonoids have been identified until now and their numbers are still increasing. All flavonoids consist of two aromatic rings (A, B) which are bound together by three carbon atoms (Figure 3). These three carbons are combined with oxygen and two carbons of the aromatic ring A to form an oxygenated heterocycle (C).

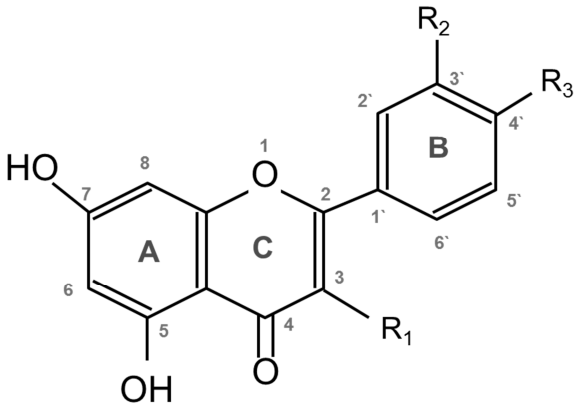


**Figure 3** General structure and numbering pattern for common food flavonoids. Adapted from Beecher (2003)

Flavonoids are divided into the subclasses flavonols, flavones, isoflavones, flavanones, anthocyanidins and flavanols (Figure 4) depending on the oxidation state of the heterocycle (Cheynier 2005, Manach et al 2004). Their immense diversity stems not only from the different oxidation states of the heterocycle, but also from the numerous substitution patterns on the aromatic rings and the wide range of glycosides. Until today, more than 80 sugars have been detected in flavonoid glycosides, and for the flavonol quercetin alone, 179 different glycosides have already been described (Watzl and Rechkemmer 2001).

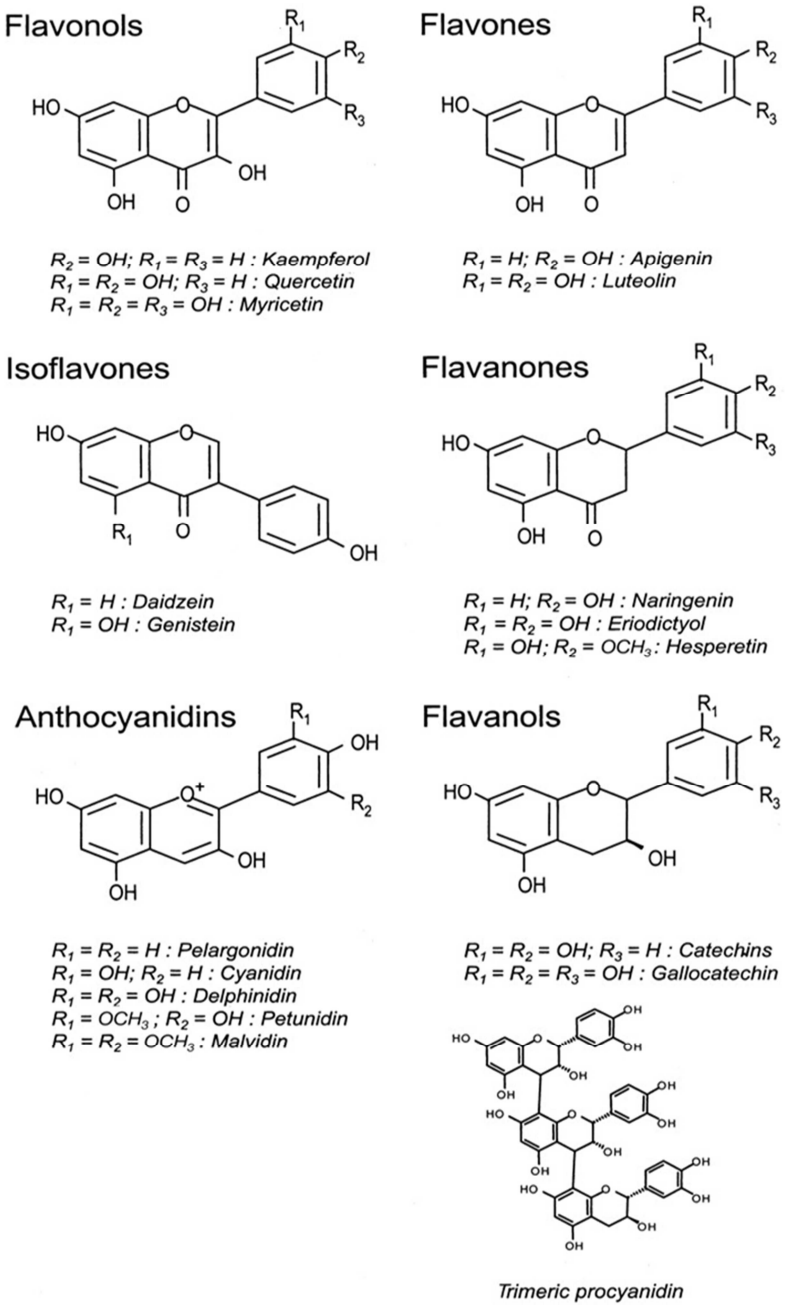


**Figure 4** Chemical structure of flavonoids (Manach et al 2004)

*Flavonols*

Quercetin, as well as kaempferol, belong to the flavonol subclass, which are the most ubiquitous flavonoids, with high concentrations in apples and onions. They usually occur in their glycosylated form, in most cases bound to a glucose moiety, and are rarely connected to other sugars such as arabinose, galactose, rhamnose and xylose (Aherne and O'Brien 2002, Bohm et al 1998).

*Flavones and Flavanones*

Flavones have the same basic structure as flavonols, only differing in a hydroxyl group at the $C_3$. The most prominent flavones are apigenin and luteolin which have their highest concentrations in citrus fruits, grapes, lettuce and celery (Amarowicz et al 2009, Bohm et al 1998).

The flavanones naringenin and hesperidin are also present in fairly high concentrations in citrus fruits (especially in oranges and grapefruits) mainly as glycosides (Dixon and Paiva 1995, Fowler and Koffas 2009).

*Isoflavones*

Isoflavones are almost exclusively found in legumes, with highest concentrations in soy beans (Crozier et al 2009). The two main isoflavones are genistein and daidzein which normally occur as their glycosides genistin and daidzin (Valls et al 2009). They are also referred to as phytoestrogens since they show estrogenic activity (Ward and Kuhnle 2010) .

*Flavanols*

Flavanols (flavan- 3- ols) often occur in combination with their oligo- and polymers also named proanthocyanidins or condensed tannins (Escarpa and Gonzalez 2001). They are termed proanthocyanidins since they react to red colored anthocyanidins when heated under acidic conditions (Cheynier 2005). The most common flavan- 3-ols are (+)-catechin/ (-)-epicatechin, (+)-gallocatechin/ (-)-epigallocatechin and (+)-afzelechin/ (-)-epiafzelechin with their oligo- and polymers procyanidins, prodelphinidins and propelargonidins, respectively (Serrano et al 2009). The stereoisomers, (-)-catechins and (+)-epicatechins are comparatively rare in nature (Crozier et al 2009). A specific characteristic is that flavanols occur in nature as aglycones (not esterified with a sugar), which is very uncommon (Escarpa and Gonzalez 2001). They are widespread in plants, high concentrations are found in tea, grapes, wine, cacao, and they are assumed to account for a significant part of the PP in the western diet (Amarowicz et al 2009, Serrano et al 2009).

*Anthocyanins*

Anthocyanins are responsible for the red, blue and purple color of fruits, vegetables and flowers. They are the methoxylated or hydroxylated glycosides of the

anthocyanidins pelargonidin, cyanidin, delphinidin, peonidin, petunidin and malvidin (Mazza 2007), with cyanidin as the most common anthocyanidin (50 %). Anthocyanidins can be differentiated by their number of hydroxyl groups, the number, nature and position of the sugar bonds, as well as the nature and number of aliphatic or aromatic acids attached to the sugars (Kong et al 2003).

### 2.1.2 Biosynthesis of different PP compounds

The shikimate pathway links the carbohydrate metabolism to the biosynthesis of aromatic compounds, leading to the production of the amino acids phenylalanine, tyrosine and tryptophan as well as to the synthesis of phenolic compounds (Figure 5) (Herrmann and Weaver 1999).

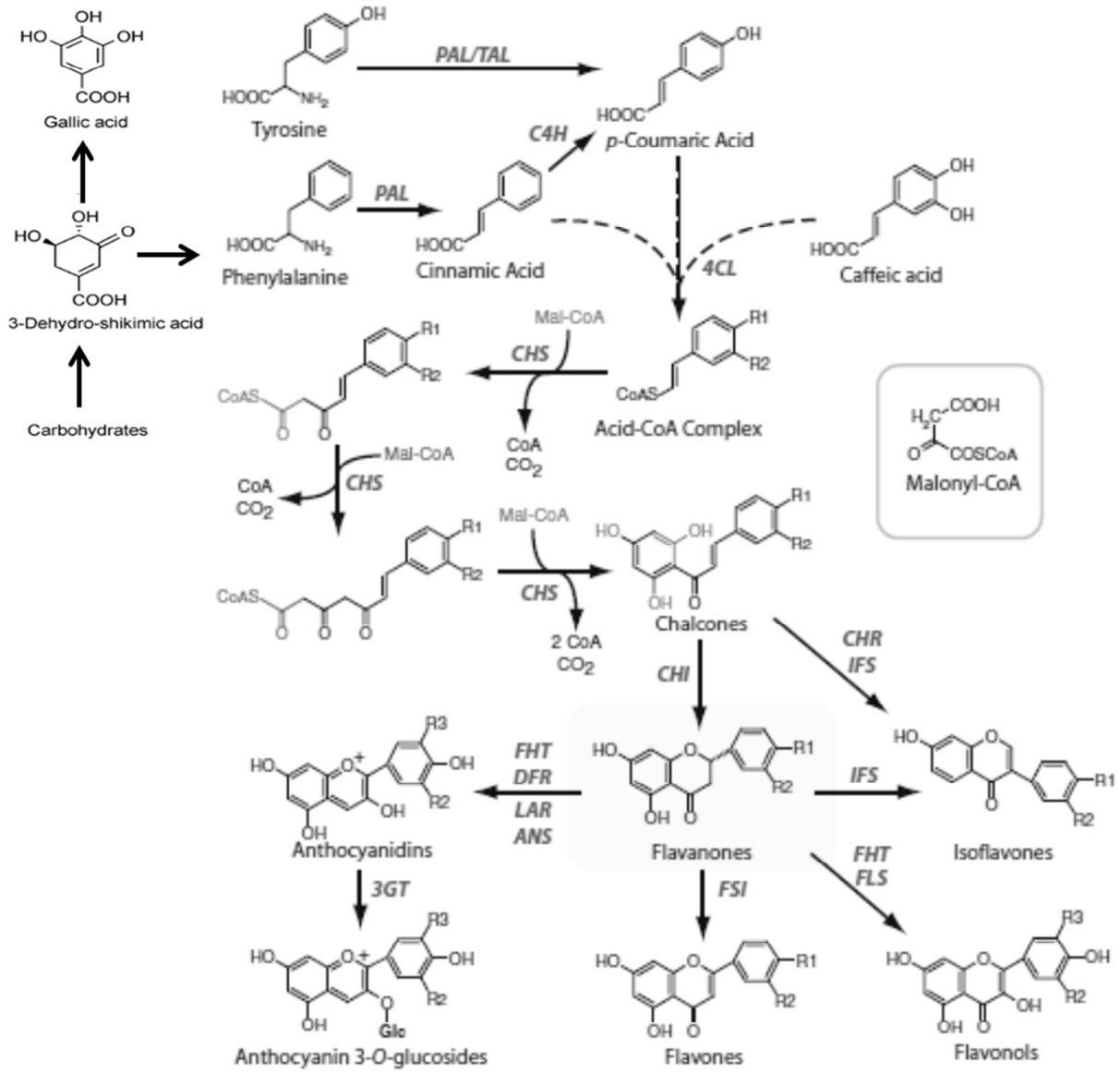


**Figure 5** Schematic diagram for polyphenol biosynthesis. The three successive carbons added from malonyl-CoA by CHS are shown in green, red, and blue. Abbreviations are in text or as follows: DFR dihydroflavanone reductase, LAR leucoanthocynanidin reductase, ANS anthocyanidin synthase, 3GT uridine, flavanone 3-glucoside transferase, FSI flanone synthase, CHR chalcone reductase, IFS isoflavanone synthase, FHT flavanone hydroxytransferase, FLS flavonol synthase. Diagram adapted from Fowler and Crozier (2009)

In a first step carbohydrates are converted to shikimic acid ($C_6$- $C_1$). Subsequently PP can either be directly synthesized from shikimic acid or via the phenylpropanoid pathway ($C_6$- $C_3$). Benzoic acids and gallic acid are directly synthesized from shikimic acid and consist of an aromatic phenyl group ($C_6$) and a single carbon ($C_1$). The simplest phenylpropanoids such as hydroxycinnamic acids consist of an aromatic phenyl group ($C_6$) and a three carbon propene tail ($C_3$). The key precursor of

phenylpropanoids is phenylalanine which is also derived from shikimic acid via the shikimate pathway; in rare cases tyrosine serves as aromatic amino acid for phenylpropanoid biosynthesis.

The phenylpropanoid pathway starts either with the formation of cinnamic acid out of phenylalanine or the formation of p- coumaric acid out of tyrosine, catalyzed by the enzymes phenylalanine ammonia- lyase (PAL) or tyrosine ammonia- lyase (TAL), respectively. This deamination step of phenylalanine (tyrosine) is regarded as the branch point between primary and secondary plant metabolism (Amarowicz et al 2009, Andersen and Markham 2006, Crozier et al 2009, Dixon et al 2002). A series of hydroxylations, methylations and dehydrations of cinnamic acid lead to a series of simple phenolic acids such as p-coumaric, caffeic, ferulic, and sinapic acids.

The major substrate for flavonoid synthesis is 4-coumaroyl-CoA which forms together with 3 molecules of malonyl-CoA the $C_{15}$ flavonoid backbone. The condensation of 4-coumaroyl-CoA and malonyl-CoA is catalyzed by chalcone synthase (CHS), leading to tetrahydroxychalcone, which is rapidly converted to naringenin by the enzyme chalcone isomerase (CHI; Andersen and Markham 2006, Dixon and Paiva 1995). The flavanone naringenin is the precursor for many flavonoids (Fowler and Koffas 2009).

These two first enzymes (CHS, CHI) of the flavonoid pathway are almost ubiquitously found in plants. However, the enzymes catalyzing further steps vary depending on the plant, thus resulting in different flavonoid patterns (Deng and Davis 2001). In general, flavonoid biosynthesis is well understood, but a detailed description would go beyond the scope of this literature review, but a recently published book is referenced here for further reading (Andersen and Markham 2006).

### 2.1.3 PP intake and major sources

The dietary intake of PP as well as their concentration in plants is difficult to estimate. Intake varies widely, depending on dietary habits and preferences. Additionally the structural diversity complicates exact measurements of their concentrations in foods as well as the calculation of daily intake. Depending on the method used, the total PP concentration or the concentration of single PP can be determined. Identification and quantification of each single PP is further complicated by their enormous number and

the fact that standards are not readily available for most of them. The following section describes the PP concentration and intake from foods most of them commonly eaten in Europe and the US. It is assumed that the major part of the daily PP intake can be attributed to the consumption of beverages such as tea, beer, coffee and wine, and that fruits and vegetables are of minor importance.

It has been shown that beverages contribute about 60 % to the daily PP intake of people living in France. Fruits and vegetables only accounted for 28 %, with an intake from fruits that was approximately 3- fold higher than from vegetables. Total PP intake was estimated to be about 1 g per day (Folin-Ciocalteau assay; Brat et al 2007). In Finland, the mean daily PP intake ranged from 2.6 g to 3 g, mainly from coffee, tea, fruits and cereals (Ovaskainen et al 2008).

#### 2.1.3.1 Beverages

##### *2.1.3.1.1 Tea*

Tea, with an average consumption of 500 ml per day and person, is the most regularly consumed beverage (after water) worldwide (Drynan et al 2010). Fresh tea leafs are very high in PP (circa 30 % of dry weight), but concentration in tea changes during processing and fermentation (Crozier et al 2009). Flavan-3-ols are the dominating PP in tea, with about 14 g/100 g dry green tea and about 17 g/100 g dry black tea. During fermentation, the content of simple flavan-3-ols (catechin, epicatechin, epicatechin-3-gallate, epigallocatechin, gallocatechin, and gallocatechin-3-gallate) strongly decreases with the transformation to more complex PP, such as theaflavins and thearubigins (Crozier et al 2009, Peterson et al 2005). The latter are the major PP in black tea and are responsible for the astringent taste and the characteristic color (Crozier et al 2009). The flavonols (quercetin, kaempferol, myricetin) and flavones (apigenin, luteolin) have been identified in both green and black tea, but at lower concentrations (Toyoda et al 1997). Black tea was also found to contain small amounts of hydrolysable tannins (Hashimoto et al 1992).

#### *2.1.3.1.2 Coffee*

Coffee beans contain at least 16 different kinds of chlorogenic acids, accounting for 6-10 % of the total bean dry weight (Clifford et al 2006). The influence of processing on the PP pattern of coffee is negligible, but losses during roasting are high. It has been estimated that one cup of coffee still contains between 20 mg and 700 mg of chlorogenic acids (Clifford 2000).

#### *2.1.3.1.3 Beer*

Mean total PP concentration in beer is about 539 mg/ L (Floridi et al 2003). Beer contains numerous phenolic compounds; about 70 % come from barley and about 30 % from hops. The estimated amount of proanthocyanidins in hops is between 0.5 %- 5 %. The PP profile of hops and barley is comparable; it only differs in the higher proportion of gallocatechin units in barley (Stevens et al 2002). In total, about 50 % of the proanthocyanidins in beer are prodelphinidins (epigallocatechin units; Gu et al 2003).

#### *2.1.3.1.4 Wine*

Red wine and white wine contain 1000- 3000 mg/L PP and 250- 1500 mg/L PP, respectively. Their content, however, strongly varies between grapes, and is also influenced by the different processing techniques (Burns et al 2000, Landrault et al 2001). The PP profile of grapes depends on several factors, such as species, variety, season, soil and climate (Jackson and Lombard 1993, Mazza et al 1999). In general, it can be stated that red wine contains high amounts of flavan3-ol monomers (catechin and epicatechin) as well as procyanidin oligomers (DP 2- 10) and polymers (DP> 10), whereas white wines are low in flavan-3-ol monomers and procyanidins are nearly inexistent (Carando et al 1999, Gu et al 2003, Landrault et al 2001). However, red wines have also been reported to contain high amounts of prodelphinidins (de Pascual-Teresa et al 2000, Gu et al 2003, Gu et al 2004). The major PP responsible for the red wine colors are anthocyanidins, which are extracted from the grape skin during processing (Landrault et al 2001, Mazza et al 1999). Furthermore, all kinds of flavonols as well as resveratrol have been detected in red wine (Burns et al 2000). The latter has been linked to the "French paradox", which is

the low incidence of coronary heart disease in the French population, despite the high consumption of dietary fat (Das and Das 2010, Renaud and Delorgeril 1992). Gallic acid and some hydroxycinnamic acids such as caffeic acid and p-coumaric acid can be found in both, red and white wine (Pereira et al 2010).

*2.1.3.1.5 Cocoa and chocolate*

Chocolate and fresh cocoa beans contain high quantities of mono- and oligomeric flavanols, mainly derivatives of catechin and epicatechin (Gu et al 2006). The oligomeric procyanidins range from dimers to decamers with highest concentrations found for di- and trimers. Also high quantities of monomers do occur (about 100 mg/100 g), with epicatechin concentrations of about 72 mg/100 g and catechin concentration of about 28 mg/100 g (Donovan et al 2006). The amounts of procyanidin oligomers are much lower in chocolate than in the cocoa bean (Gu et al 2006, Hammerstone et al 1999). Fermentation and processing lead to a transformation of PP to insoluble polymeric compounds (Crozier et al 2009).

2.1.3.2 Fruits

*2.1.3.2.1 Apples, Pears and other fruits*

Fruits, especially apples, with PP concentrations ranging from 231- 488 mg/ 100 g, make a large contribution to the daily PP consumption in the Western world (Crozier et al 2009). Together with potatoes, they accounted for 47 % of the daily fruit and vegetable PP intake in France (Brat et al 2007). Apples contain various PP, such as proanthocyanidins (mainly epicatechin mono-, di- and oligomers) chlorogenic acids (mainly 5-caffeoylquinic acid) and flavonols (e.g. quercetin conjugates; Crozier et al 2009, Lees et al 1995, Santos-Buelga and Scalbert 2000).

The PP pattern of pears is comparable to apples, with a high percentage of proanthocyanidins (average degree of polymerization 13- 44; mainly consisting of epicatechin units; Ferreira et al 2002).

Stone fruits such as peaches, nectarines and plums have comparable PP patterns (hydroxycinnamic acids, procyanidins, flavonols and anthocyanins) in similar

concentrations, with procyanidins the dominating PP in almost all varieties (Tomas-Barberan et al 2001).

Citrus fruits are the major dietary source of flavanones, mainly located in the tissue of the fruits, but also in considerable amounts present in the pulp. They occur as glycosides of the two flavanones naringenin and hesperidin, where hesperidin is dominant in sweet orange, lemon, lime and mandarin, and naringenin in sour orange and grapefruit (Tomas-Barberen and Clifford 2000).

#### 2.1.3.3 Vegetables

##### *2.1.3.3.1 Carrot, onions, broccoli, spinach, eggplant and zucchini*

Highest concentrations can be found in spinach (100- 690 mg/100 g), eggplants (340 mg/100 g) and broccoli (320 mg/100 g; Bunea et al 2008, Gillooly et al 1983), whereas carrots and zucchini belong to the vegetables with lowest PP concentrations (Cieslik et al 2006, Gillooly et al 1983).

Very high concentrations (up to 100 mg/ 100 g) of chlorogenic acids (5-caffeoylquinic acid) can be found in eggplants (Luthria et al 2010), which also contain considerable amounts of delphinidin derivatives, which belong to the anthocyanins and are responsible for the eggplants color (Wu and Prior 2005). In contrast, 75 % of the phenolic fraction of broccoli is represented by two flavonol glycosides (quercetin and kaempferol glycoside) and four hydroxycinnamic acid esters (Plumb et al 1997). Onions are considerable providers of flavonols in the western diet, with quercetin concentrations up to 63mg/ 100g (Crozier et al 1997). Spinach, contains the flavonols patuletin, quercetin as well as kaempferol (free and conjugated; Gil et al 1999, Stewart et al 2000). The flavone apigenin however is the major PP in spinach (17mg/100g; Dehkharghanian et al 2010). Onions contain considerable amounts of hydroxybenzoic acids, which are usually present in plants only in low concentrations (Shahidi and Naczk 1995).

#### 2.1.3.4 Beans

Common beans contain a wide range of PP including phenolic acids, proanthocyanidins, anthocyanidins as well as flavonols, the latter three being responsible for bean pigmentation (Caldas and Blair 2009). PP content and profile between beans differ strongly and is determined by the bean variety and seed color (Beebe et al 2000). As quantified by Folin Ciocalteau method, white beans have by far the lowest PP concentration, ranging from 40- 80 mg PP/ 100 g beans and red colored beans tend to have highest PP concentrations with up to 800 mg/ 100 g (expressed in gallic acid equivalents; Tajeri Foman 2006). With the exception of white beans, variations in PP level within a single color class however can be higher than between the different color classes (Beebe et al 2000).

Condensed tannins (proanthocyanidins), the major PP in colored beans, are primarily located in the bean hull (Anton et al 2008, Reddy et al 1985). Diaz and colleagues (2010) screened about 250 beans and measured proanthocyanidin levels in the seed coat with an acid butanol assay according to Caldas and Blair (2009). Concentrations ranged from 10 % to 30 % of seed coat weight, with an overall average of about 20 %. Since bean hulls account for approximately 7 % of total bean weight, the amount found in hulls was equivalent to total proanthocyanidin concentrations of up to 2 g/ 100 g bean. Lower proanthocyanidin concentrations were reported from authors using a Vanillin assay for quantification. Beninger and colleagues (2005) found concentrations ranging from 0.2 to 1.1 g/ 100 g bean, similar to Maldonado and co-workers (1996) who found concentrations between 0.25 and 0.95 g/ 100 g beans. As identified by mass spectrometry, the majority of bean proanthocyanidins are procyanidins (catechin and epicatechin units; Aparicio-Fernandez et al 2005, Beninger et al 2005). Prodelphinidins ((epi)-gallocatechin), although in lower concentrations than procyanidins have been reported by Aparicio-Fernandez and colleagues (2005), whereas Gu and co-workers (2004) detected no prodelphinidins in beans. Researchers investigating flavan-3-ol bean oligomers (up to 6 units) reported the most prevalent oligomers were dimers (Aparicio-Fernandez et al 2005). Beninger and colleagues (2005) however looked at the whole range of condensed tannins and found more than 80 % polymers of greater than 10 subunits. Other authors (Gu et al 2003, Gu et al 2006) reported average DPs ranging from 7 to 12 units, depending on the bean variety. The DP of bean proanthocyanidins is comparable to red wine (7

units), sorghum (8 units) and cocoa, but proanthocyanidins in beans stand out due to their high concentration in epi(afzelechin) which accounts for up to 15 % of proanthocyanidin units.

Flavonol glycosides such as quercetin and kaempferol, are exclusively present in colored beans (Lin et al 2008). Kaempferol is the major flavonol with concentrations ranging from 5 to 15 mg/ 100 g bean (Beninger et al 2005, Espinosa-Alonso et al 2006). However, flavonol as well as proanthocyanidin concentration in beans, is influenced by the seed storage period. The post-harvest aging process is associated with depolymerization of proanthocyanidins and results in higher concentrations of monomers to decamers. Storage additionally leads to a reduction in flavanol concentration (Beninger et al 2005).

Intensively colored beans are rich in anthocyanins which are also exclusively located in the seed coat. Black beans were found to contain up to 218 mg/ 100 g anthocyanins, with highest concentrations in delphinidin 3- glycoside (56 %), but no anthocyanins were found in white beans (Choung et al 2003, Takeoka et al 1997). Wu and colleagues identified delphinidin-, malvidin- and petunidin glycosides in black beans, whereas red beans contained cyanidin- and pelargonidin glycosides (Wu and Prior 2005).

Beans also contain numerous phenolic acids, including hydroxybenzoic, p-coumaric, caffeic and ferulic acids (Laparra et al 2008), but at very low concentrations. The most abundant phenolic acid in colored beans is ferulic acid (3 mg/ 100 g), whereas the most abundant phenolic acid in white beans is hydroxybenzoic acid (0.7 mg/ 100 g; Espinosa-Alonso et al 2006, Laparra et al 2008).

In conclusion, PP concentrations in beans vary strongly depending on genotype and color. In addition results differ strongly due to the use of different chemical assays for their quantification (Carmona et al 1991, Vanderpoel et al 1991). Espinosa-Alonso and colleagues (Espinosa-Alonso et al 2006) determined total PP concentration with Folin Ciocalteau method and proanthocyanidin concentration with Vanillin assay in the same beans. They found total PP concentrations ranging from 90- 210 mg/ 100 g and proanthocyanidin concentrations ranging from 950 mg- 3600 mg/ 100 g.

#### 2.1.3.5 Sorghum

Sorghum has a comparable PP pattern to beans. Total PP concentration is depending on color as well as on genotype. Red colored sorghum has been shown to be highest in PP with 300- 900 mg /100 g, as quantified by Folin Ciocalteau. However, variation is high, also depending on the thickness of the pericarp (Dykes et al 2005). Proanthocyanidins are only present in colored sorghum varieties (Dykes and Rooney 2006), with highest concentrations in the range of 1200- 1500 mg/ 100 g, as quantified by vanillin assay (Dykes et al 2005). Similar to beans, more than 80 % of the proanthocyanidins present in sorghum have a DP >10 (Awika et al 2003). There is some evidence that compared to beans certain sorghum varieties have higher concentrations of galloyl group bearing PP such as prodelphinidins (Towo et al 2003), but results are inconsistent (Gu et al 2003, Gu et al 2004). The highest anthocyanin concentrations (luteolinidin and apigeninidin) have been reported from black sorghum varieties (Awika et al 2005).

The most abundant phenolic acid is ferulic acid with concentrations of up to 30 mg/ 100 g (Hahn et al 1983).

### 2.1.4 Absorption of PP

Since PP are a very heterogeneous class of substances, some of them are more bioavailable than others, with absorptions ranging from below 1 % to 43 %. Those most abundant in our diet are not necessarily those that have the best bioavailability. But sufficient absorption is indispensable to potentially exert biological effects (Manach et al 2005). The absorption of PP depends on various factors, which can be roughly divided into 5 categories: 1) external factors (i.e. sun exposure effects concentration and pattern of PP in the plant); 2) food processing related factors (i.e. cooking and other thermal treatments); 3) food related factors (presence of compounds affecting PP absorption such as fiber and fat, proteins and other PP); 4) PP related factors (chemical structure and concentration); 5) host related factors (intestinal factors such as microflora or systemic factors such as gender or age) (D'Archivio et al 2010).

The focus of this review will be on categories 4 and 5, since they are considered to be more important.

The absorption of PP occurs in the small intestine as well as in the colon after microbial degradation. The majority of PP is however fermented by the human gut microbiota (Selma et al 2009).

#### 2.1.4.1 Absorption of intact PP structures

The absorption of most flavonoids takes place in the small intestine and to a lesser extent in the colon. This has been shown in several human bioavailability studies. Glucose conjugated quercetin from onions was quickly absorbed, with quercetin peak plasma levels after 20 min (Hollman et al 1997), and also genistein and daidzein from a soybean flour based meal appeared in plasma 30 min after consumption (King and Bursill 1998). The plasma levels of malvidin from red wine (Bub et al 2001) and catechins from green and black tea extracts (Leenen et al 2000) reached a maximum within the first hour after consumption.

The time to reach maximal plasma levels is influenced by the food matrix (interactions with proteins and polysaccharides), PP structure and PP concentration, and in some cases maximal plasma levels were reached only after 4 hours (Mazza et al 2002). It has been shown that the absorption of flavan-3-ols depends on their degree of polymerization and structure (Manach et al 2005), and there is some evidence that only small amounts of proanthocyanidins are absorbed and detected in plasma. Holt and co-workers found a 100- fold lower proanthocyanidin plasma concentration (41 nmol/ L) compared to the concentration of their monomers. Interestingly, epicatechin was much better absorbed than catechin. Catechin only accounted for about 3 % of the epicatechin plasma concentration, although their ratio was 1:1 in the administered test meals. The authors concluded from the results that most likely procyanidin dimers and oligo/polymers were degraded to their monomer (epicatechin) in the colon by microbiota before absorption (Holt et al 2002).

Most flavonoids such as catechin, quercetin, genistein and hesperidin do not appear in plasma as the glycoside or in their free form. The majority found in the blood are methylated, sulfated or glucuronidated derivatives (Day et al 2001, Natsume et al 2003). This is not the case for anthocyanidins which are usually found in plasma and urine as unchanged glycosides, though in low concentrations (Manach et al 2005). Little is known about the absorption of phenolic acids. Chlorogenic acid is mainly absorbed in the colon after hydrolyses by microbial esterases (Gonthier et al 2003),

whilst the absorption of ferulic and sinapic acid most likely occurs in the small intestine (Kern et al 2003). Human studies focusing on the absorption of gallic acid found a rapid uptake and high plasma values (2.1 µmol/ L) after consumption of tea (Shahrzad et al 2001).

Two different mechanisms involved in the uptake of PP in the small intestine are discussed in literature. First, it is assumed that uptake is catalyzed by lactase phlorizin hydrolase (LPH), an enzyme found in the brush border of the mammalian small intestine. LPH, a beta-glycosidase also involved in lactose metabolism, hydrolyzes flavonoid glycosides and enables the uptake of the hydrophobic aglycones. Uptake of flavonoid glycosides by passive diffusion is most unlikely due to their hydrophilic character (Day et al 2000b).

The second suggested mechanism of PP absorption involves the hydrolysis of the flavonoid glycosides by cytosolic beta-glucosidase (CBG), which is located in the epithelial cells. This requires the transport of the glycosylated, hydrophilic flavonoids into the enterocyte (Gee et al 2000), which is probably performed by the sodium-dependent glucose transporter (SGLT1). This theory is supported by a study conducted by Hollman and colleagues in ileostomy subjects. They found that absorption of quercetin-β-glucosides from onions was 52 %, whereas absorption of quercetin without its sugar moiety was only about 20 %, suggesting that the sugar moiety of quercetin glycosides is important for their absorption (Hollman et al 1995).

Other studies demonstrated that PP inhibited $Na^+$ dependent (SGLT1) and $Na^+$ independent (GLUT) glucose uptake in a competitive manner, which is a further indicator for the above mentioned theory. Somehow, LPH and CBG are involved in PP uptake, but their relative contribution remains to be clarified (Cermak et al 2004, Walgren et al 2000, Wang et al 2008).

Once absorbed, PP appear in plasma bound to proteins, in most cases to serum albumin, and undergo the enterohepatic circulation. They are transported to the liver, where they are mainly conjugated and from there to the tissues (Dangles et al 2001, Manach et al 2004, Yang et al 2001). Their appearance in numerous tissues of mice such as brain, heart and kidney has been demonstrated (Suganuma et al 1998). Their conjugation is part of the body's detoxification process. For example, glucuronidation increases the solubility and molecular weight of PP and consequently, is necessary for their excretion in the bile (Day et al 2000a).After secreted via the biliary route into the duodenum (large heavily conjugated PP) some

PP are reabsorbed as aglycones. Smaller conjugates might also be excreted in the urine (Manach et al 2004, Yang et al 2001). The exact metabolic pathway of PP is yet unclear. It is assumed that a complex system of carriers and enzymes is involved in the regulation of PP uptake as well as in PP metabolite production and release by the hepatocytes (Manach et al 2004).

#### 2.1.4.2 Microbial PP degradation and absorption of metabolites

PP not absorbed in the small intestine reach the colon where they are enzymatically degraded by human gut microbiota (Figure 6). Gut microbiota have the ability to hydrolyze glycosides, glucuronides, sulfates, amides, esters, and lactones, but they also carry out reactions including ring-cleavage, reduction, decarboxylation, demethylation, and dehydroxylation (Hanhineva et al 2010). A small fraction of the generated aglycones are directly absorbed; but the majority is further degraded to simple phenolic acids and nonphenolic aromatic metabolites before absorption. Depending on the flavonoid, the composition of the generated phenolic acids differs strongly (Aura et al 2005, Deprez et al 2000). However, the metabolizing of PP by gut microbiota is of great importance since the compounds produced could be highly bioactive and could effect human health (Lee et al 2006, Selma et al 2009).

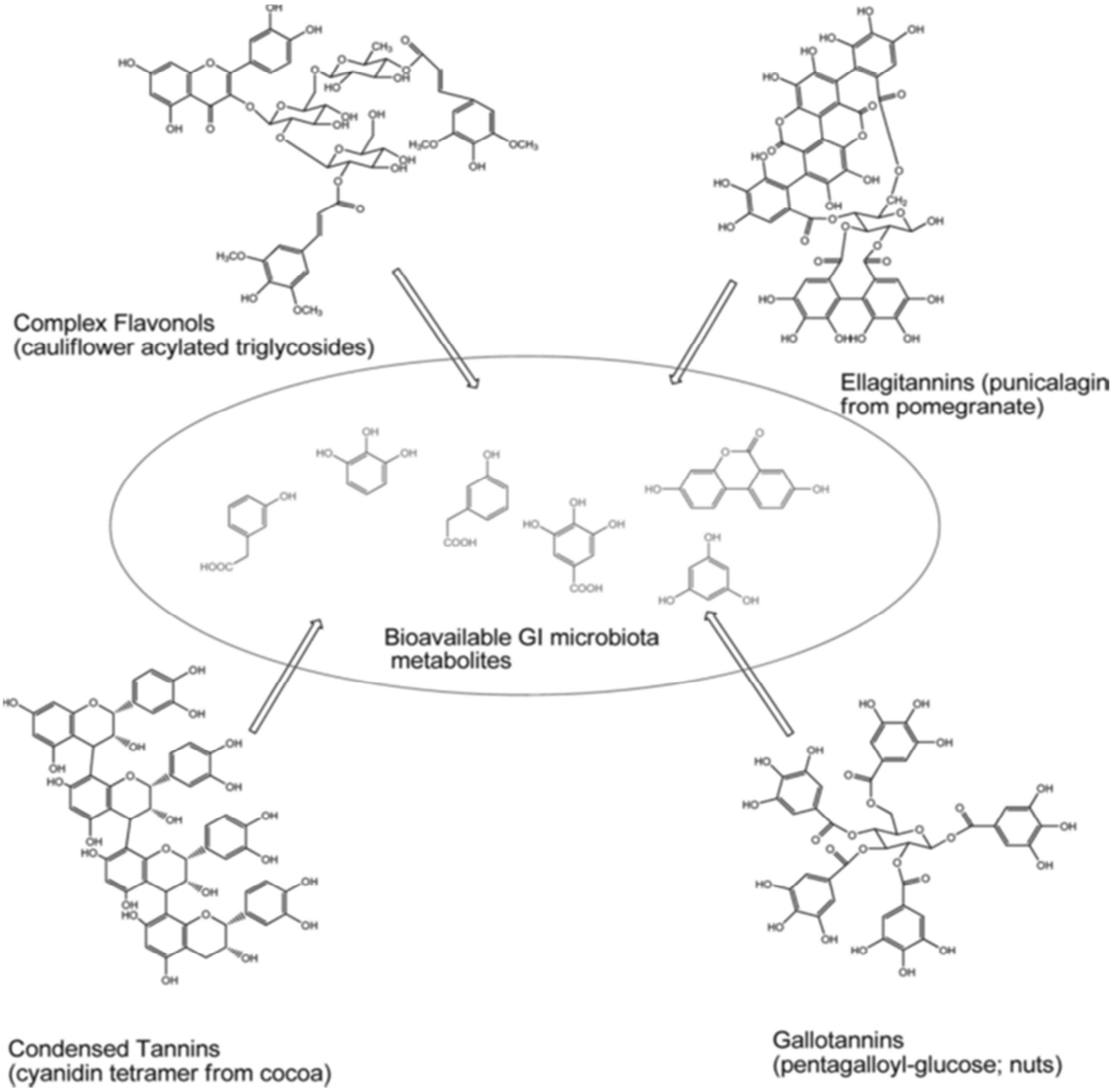


**Figure 6** Complex dietary PP are converted to highly bioavailable compounds by human gut microbiota (Selma et al 2009).

### 2.1.5 Positive health impacts of polyphenols

#### 2.1.5.1 Cancer

PP possess the optimal structure for free radical scavenging and their antioxidative capacities have largely been demonstrated. There is epidemiologic evidence that diets with a high proportion of antioxidant rich fruits and vegetables reduce the risk of

many cancers (Dai and Mumper 2010). Extracts of several fruits have been shown to suppress the growth of human oral, breast, colon and prostate tumor cells *in vitro* in a dose dependent manner (Seeram et al 2006, Zhang et al 2008). Similar results have been reported from studies with wine extracts and isolated PP (resveratrol, quercetin, catechin, epicatechin; Damianaki et al 2000, Kampa et al 2000). The anticancer effect of several anthocyanins has been demonstrated in rats and mice (Ding et al 2006, Lala et al 2006), whereas the protection against oxidative stress and improvement of oxidative status has been shown in several human short term studies (1 dose) with PP- rich foods such as red wines, strawberries (Cao et al 1998) and chocolate (Rein et al 2000a). However, results of human studies are still inconsistent (Halliwell et al 2000, Scalbert et al 2005, Yang et al 2001), and more well designed studies are necessary to clarify the impact of PP on cancer and other degenerative diseases. In general, it has to be noted that numerous *in vitro* studies investigated the biological activity of different PP and that most health impact assumptions rely on these results. Yet, many of the *in vitro* studies have no physiological relevance, since the administered dose exceeds by far the concentrations in the plant and later in the human host. Moreover, as already described above, absorption goes along with conjugation and metabolism of PP. The compounds investigated in most *in vitro* studies had the same structure as they occur in plants (aglycones or their sugar conjugates) and not as their active metabolites, most likely not being relevant to the *in vivo* situation. To clearly assess the impact of PP on cancer, more studies taking into consideration the PP concentration and form, are necessary.

#### 2.1.5.2 Cardiovascular diseases

The relationship between PP and cardiovascular disease has been the subject of many epidemiological studies. Results are conflicting, but there is some evidence that individuals with high flavonoid intake have a reduced risk of cardiovascular disease. It has been shown in a case control study that subjects consuming more than one cup of tea per day had a 44 % reduction in cardiovascular risk (Sesso et al 1999). The same effect has been demonstrated in a further study where moderate and heavy tea drinkers had 31 % and 39 % reduction in cardiovascular risk, respectively (Mukamal et al 2002). There is evidence from a meta-analysis conducted in 2002 that drinking red wine reduces the overall vascular risk by 32 %,

whereas cardiovascular mortality was only significantly reduced in women (Di Castelnuovo et al 2002). The positive effect of red wine on cardiovascular disease however might be attributed to the presence of resveratrol (Das and Das 2010). Cocoa is another flavonoid-rich food that may have health benefits. The consumption of 100 g dark chocolate for 14 days significantly reduced blood pressure (Taubert et al 2003). Other studies demonstrated that cocoa improved the dilatation of the brachial artery (Heiss et al 2003), inhibited platelet aggregation (Rein et al 2000b) and may have favorable effects on cardiovascular mortality (Grassi et al 2009).

#### 2.1.5.3 Impact of PP on carbohydrate metabolism

Since PP inhibit the glucose absorption rate by competing with glucose for SGLT1 and GLUT they have the ability to counteract postprandial hyperglycemia. There is also some evidence that PP positively influence the carbohydrate metabolism by stimulating insulin secretion from the β- cells of the pancreas, suppressing glucose release from liver storage and improving glucose uptake in peripheral tissue. Yet, most data is derived from *in vitro* and animal studies and human data is still lacking (Hanhineva et al 2010).

#### 2.1.5.4 Bone health

Recent research has suggested that tea consumption is positively correlated with bone mineral density, whose rapid decrease after menopause is a major risk factor for hip fractures (Hegarty et al 2000, Kara et al 2007).

#### 2.1.5.5 Estrogens

Phytoestrogens potentially exhibit favorable estrogenic characteristics, although their estrogenic activity is weak compared to endogenous estrogens. It is suggested that phytoestrogens might protect against cardiovascular disease (Tham et al 1998) and exhibit hypocholesterolemic effects (Sirtori et al 1998). There is also some evidence that phytoestrogens ingested with food may have beneficial effects in the prevention of breast cancer and for the treatment of post-menopausal problems. Their structure

is similar to tamoxifen, a compound with anti-estrogenic characteristics serving as a preventive of breast cancer (Tham et al 1998).

#### 2.1.5.6 Impact of PP on gut health

PP serve the human gut microbiota as substrate for growth and activity, which leads to a modulation of bacterial composition in the gut. Among others, catechin has been shown to selectively stimulate the growth of bifidobacteria spp. and thus, exhibits prebiotic potential (Tzounis et al 2008). Moreover, tea polyphenols suppressed the growth of potential pathogens (Lee et al 2006).

### 2.1.6 Negative health impacts of polyphenols

#### 2.1.6.1 Interaction of polyphenols with iron

The structure of certain PP enables them to form complexes with proteins (Naczk et al 1996, Naczk et al 2001), polysaccharides (Barahona et al 1997) and metallic ions (McDonald et al 1996), especially iron and to influence the absorption of these nutrients.

##### *2.1.6.1.1 The nature of iron polyphenol complexes*

Depending on their structure, PP can form non-absorbable complexes with iron in the intestinal tract. Data suggests that PP with an *ortho*-dihydroxy (catechol) or trihydroxy-benzene group (galloyl) such as proanthocyanidins (catechol groups and galloyl groups) and hydrolyseable tannins (galloyl groups) are the most potent iron absorption inhibitors (Brune et al 1989a, Hurrell et al 1999). Brune and colleagues (1991) looked more closely at this topic and they developed a spectrophotometric assay to measure iron binding phenolic groups in food. They were able to measure blue-colored iron-trihydroxybenzene (galloyl groups) complexes and green-colored iron-dihydroxybenzene (catechol groups) complexes, demonstrating that both groups can bind iron.

PP: iron complexes are chelates, since PP are bidentate ligands and therefore bind iron through two sites (Figure 7). The phenolic group is only a strong ligand for iron if

it is in the state of deprotonation and with it generates a highly charged oxygen center. A high pH favors deprotonation and complex formation, but is has been shown that both can already occur at physiological pH between 5 and 8 (Hider et al 2001, Purawatt et al 2007).

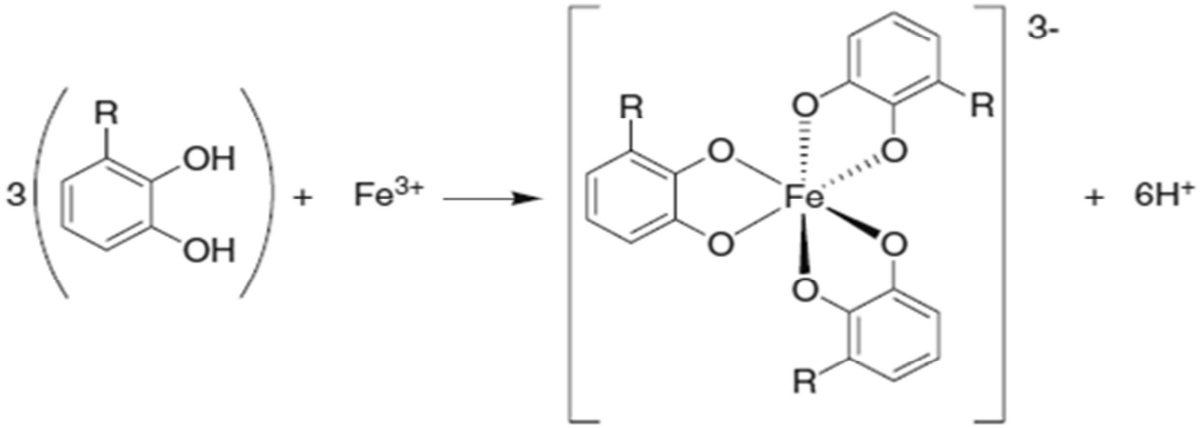


**Figure 7** Expected octahedral coordination geometry of general iron–PP complexes. Gallols, R=OH; catechols, R=H. Coordination requires deprotonation of the polyphenol ligands (Perron and Brumaghim 2009)

The complexes formed between $Fe^{3+}$ and PP are very stable, whereas $Fe^{2+}$ and PP form much weaker complexes. Due to the preferred octahedral geometry (six atoms or groups of atoms are arranged around a central atom) of metal ions, it is proposed that each iron binds up to three catechol or galloyl groups, equivalent to a PP:iron binding ratio of 3:1 (Figure 7). However, it has to be taken into consideration that several factors such as pH, ratio of metal to PP in solution and the varying PP structures have an influence on the binding ratio (Perron and Brumaghim 2009).

Flavonols such as quercetin and myricetin can bind iron with the galloyl/catechol group (A) additionally between the oxygen of the 4-position and the hydroxyl group at 3-position (B) or at 5-position (C), but with much less affinity to iron than the galloyl and catechol group (Figure 8). However, if a phenol group is conjugated by a sugar moiety the dissociable proton is lost and it no longer binds to iron (Hider et al 2001).

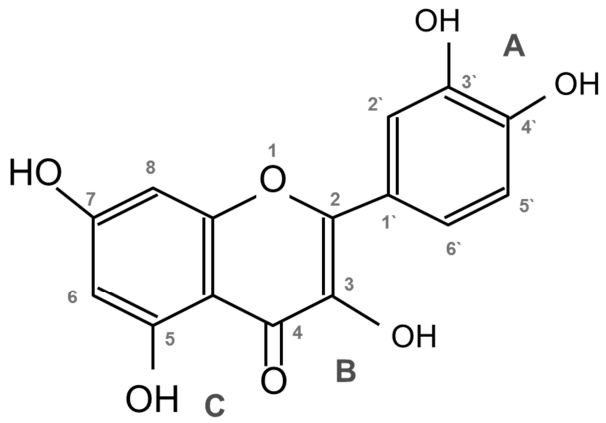


**Figure 8** General structure of flavonols and possible binding sites for iron (Hider et al 2001)

*2.1.6.1.2 Iron isotope absorption studies in human subjects*

The effect of PP from different foods on human iron absorption has intensively been investigated several times over the last decades.

*Beverages*

Disler and colleagues (Disler et al 1975) were the first showing that tea has a negative impact on human iron absorption even in the presence of ascorbic acid. Two hundred ml tea, prepared from 5 g dry tea, was served with different meals and a decrease in iron absorption of up to 80 % was observed. Hurrell and colleagues (1999) investigated the inhibitory effect of different beverages on human iron absorption. They reported that 200 mg of PP (GAE; quantified with Folin Ciocalteau) from herb teas and black tea reduced iron absorption from a bread meal by 60- 80 % and that 116 mg of cocoa PP reduced iron absorption by 70%. Also red wine, containing high quantities of proanthocyanidins (total PP concentration about 2- 3 g/ L) has been reported to be inhibitory when served with a simple bread meal. Iron absorption was 2- 3 times lower compared to white wine (total PP concentration 0.19 mg/ L) and 3- 4 times lower compared to water. Iron absorption with white wine did not differ significantly from water (Cook et al 1995). Serving red wine with a composite meal did not affect iron absorption, whereas tea and coffee reduced iron absorption by 62 % and 35 %, respectively (Hallberg and Rossander 1982).

Looking more closely at the studies comparing the effect of different beverages on iron absorption from a simple bread meal led to the conclusion that black tea PP are more inhibitory than PP from herb teas, cocoa and wine, most likely due to the higher concentration of galloyl group containing PP (Figure 9; Hurrell et al 1999).

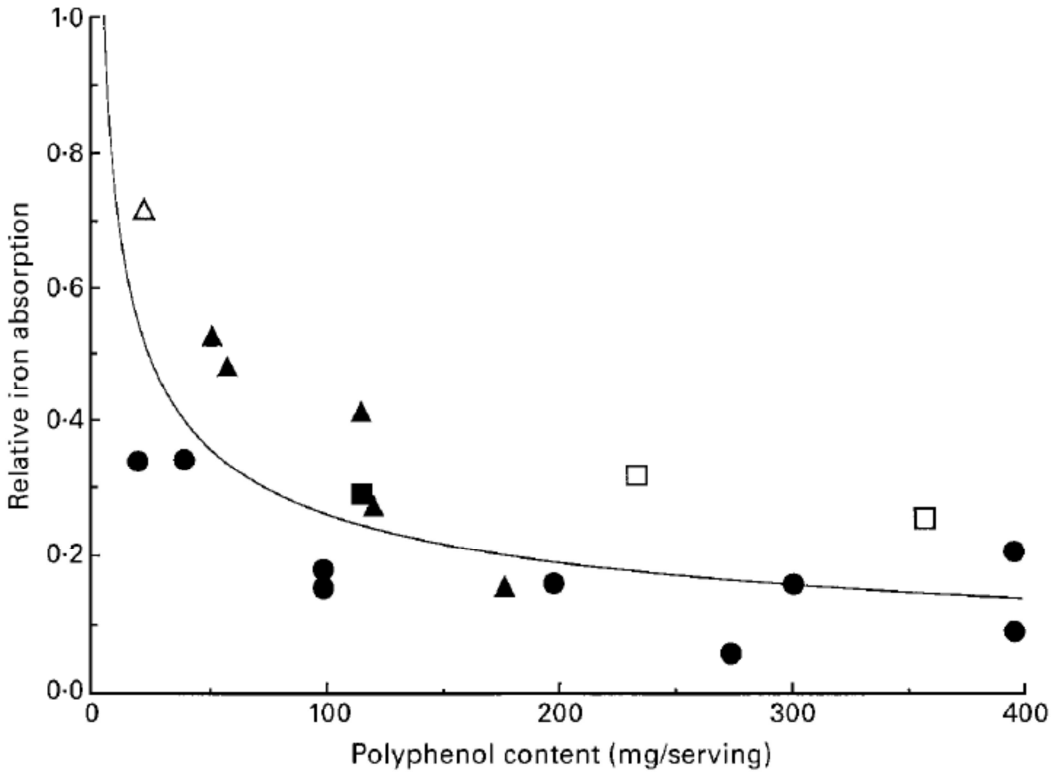


**Figure 9** Variation in relative iron absorption from a bread and beverage meal according to the polyphenol content of the beverage. Relative iron absorption is defined as iron absorption (% dose) from a bread meal consumed together with a beverage relative to iron absorption in the same subject from a bread meal consumed with water. Black tea (black dot); herb teas (black triangle); white wine (white triangle); cocoa (black square); red wine (white square). Values for red and white wine are taken from Cook and colleagues (Cook et al 1995, Hurrell et al 1999)

*Vegetables*

Various vegetables have been reported to be inhibitory for iron absorption. Tuntawiroon and co-workers (1991) looked at non-heme iron absorption from typical Southeast Asian meals based on vegetables. They found a dose dependant effect of PP on iron absorption. 150 mg of PP expressed as tannic acid equivalents reduced iron absorption by about 60 %, doubling tannic acid concentration resulted in a reduction of 80 %.

Gillooly and co-workers (1983) investigated the effect of different vegetables with different PP concentrations on iron absorption in a series of radio iron isotope studies. They showed that wheat germ, aubergine, butter beans, spinach, brown lentils, beetroot greens and lentils are strong inhibitors of iron absorption, whereas broccoli, cauliflower and sauerkraut slightly enhanced iron absorption. Measuring PP concentration in vegetable revealed a strong inverse correlation between total PP content of vegetables and iron absorption.

*Sorghum*

The strong inhibitory effect of sorghum PP on iron absorption has been observed in several absorption studies (Gillooly et al 1984, Hurrell et al 2003) and recently obtained data suggests that 162 mg sorghum PP reduce iron absorption by 68 % (unpublished data Cercamondi and colleagues).

*Spices*

PP from chilli and oregano decrease iron absorption in a similar way to beverages (Brune et al 1989a, Tuntipopipat et al 2006), whereas the effect of rosemary is only moderate (Samman et al 2001). Tuntipopipat and colleagues (2006) compared iron absorption from test meals containing either turmeric or chili, both spices being a source of PP. Despite the higher amount of phenolic compounds in the turmeric meal, iron absorption was not decreased, whereas 25 mg of chili PP (GAE; quantified with Folin Ciocalteau) reduced iron bioavailability by 38 %. The reasons for the observed difference are not totally clear, but it is suggested that iron absorption from chili was reduced due to its higher concentration of quercetin. However, the authors concluded that not only PP quantity, but quality effects iron bioavailability as well and that PP from different foods have different iron binding properties.

*Individual PP compounds*

Brune and colleagues (1989a) looked at the inhibitory effect of different PP compounds on iron absorption. 5 mg of tannic acid added to a non- inhibitory test meal already reduced iron absorption by 20 %; 25 mg by 67 % and 100 mg by 88 %. The same inhibitory effect was also observed with gallic acid and chlorogenic acid, although the latter was less inhibitory. There was no effect on iron absorption when catechin was added to the test meals. Catechin is a flavanol bearing a catechol

group and therefore, similar to chlorogenic acid, is expected to bind iron and inhibit it from absorption. The authors of that study assumed that they saw no effect, because proanthocyanidins such as catechin are poorly water soluble and therefore do not form complexes with iron in the intestinal lumen (Brune et al 1989a). However, it has been shown that iron absorption is strongly inhibited by tea mainly containing proanthocyanidins, which, in addition, have been shown to be highly water soluble. Tea contains large quantities of epigallocatechin gallate, epicatechin gallate, gallocatechin, epigallocatechin and catechin (Drynan et al 2010), some of them having galloyl as well as catechol groups. Until today there is no definite answer as to whether PP with catechol or galloyl groups or both inhibit iron absorption from tea, which reflects quite well the complexity of possible PP iron interactions.

*2.1.6.1.3 Recent Caco-2 cell studies on the inhibition mechanism*

It is commonly assumed that PP inhibit iron absorption by binding iron and forming insoluble unabsorbable complexes. But researchers recently suggested that PP enhance intestinal iron uptake and that inhibition is due to decreased basolateral iron exit (Kim et al 2008). They conducted a caco-2 cell study looking at the influence of grape seed extract (GSE) and epigallocatechin-3-gallate (EGCG) on iron absorption. Apical iron uptake was significantly increased by adding GSE and EGCG to caco-2 cells which could probably be attributed to reductive effect of EGCG and GSE ($Fe^{3+} \rightarrow Fe^{2+}$). Consequently, iron concentration in the enterocyte was high, but basolateral transport was reduced. The researchers suggested that PP increased iron uptake by an unknown passive transport way, forming membrane permeable complexes with the iron. They further concluded that these complexes were not able to exit the basolateral membrane of the enterocyte. A recently conducted study revealed that the inhibitory effect of PP on basolateral iron exit is dose dependent and can be overcome by ascorbic acid (Kim et al 2011). The same researchers observed similar, but less pronounced effects in another study, this time with heme iron (Ma et al 2010).

It has to be noted that, if PP increase apical iron absorption in humans, this can only be for monomers which are absorbed. Most PP in foods are more complex compounds and would still be expected to decrease iron absorption by forming insoluble complexes.

*2.1.6.1.4 Factors influencing the PP: iron complex formation*

Several researchers investigated the influence of other compounds on PP- iron interaction. Ascorbic acid in particular counteracts the complex formation of PP and iron. A strong impact of ascorbic acid on iron- PP interaction was observed by Siegenberg and colleagues (1991). They added different amounts of tannic acid to a bread roll meal. 12 mg tannic acid already reduced iron bioavailability by 30 %, and 50 mg by 70 %. Ascorbic acid dissolved in water was able to overcome the inhibitory effect, when consumed with the meal. The authors suggested that 50 mg ascorbic acid is sufficient to restore iron absorption from a meal containing > 100 mg tannic acid. These results were supported by an *in vitro* study conducted by South and Miller (1998), were ascorbic acid prevented the complex formation between tannic acid and iron. Ethylenediaminetetraacetic acid (EDTA) is widely accepted as an enhancer of iron absorption, and EDTA iron compounds are frequently used as fortificants due to their high bioavailability (Davidsson et al 2005). However, it has been shown that black tea, high in PP, reduced iron absorption from Fe(III) EDTA 7-fold from 19.2 % to 2.8 %, but bran, containing considerable amounts of PA, had no effect on iron absorption from Fe(III) EDTA in humans (Macphail et al 1981). The results surprisingly suggest that EDTA only effectively protects iron absorption against PA but not against PP. The results are supported by another study also looking at the effect of tea on iron absorption. This study showed that NaFeEDTA only partly overcomes the inhibitory effect of PP. Iron absorption from NaFeEDTA was reduced from 11.5 % to 1.8 % after adding tea, compared to iron absorption from ferrous sulfate which was reduced from 5.7 % to 1.03 % with tea (Hurrell et al 2000).

The effect of different compounds on iron- PP complexes and on the formation of these complexes has been investigated *in vitro* with a spectrophotometric method (South and Miller 1998). When EDTA was added to the iron solution prior to tannic acid, no PP- iron complexes were formed. Adding EDTA to an iron tannic acid solution decreased the concentration of PP- iron complexes over time towards zero, indicating that the Fe-EDTA complex was more stable than the Fe-tannic acid complex. Ascorbic acid was less effective than EDTA. Adding ascorbic acid to the iron solution prior to tannic acid almost prevented complex formation between the latter two compounds, although a small increase was detected over time. But adding

ascorbic acid to a tannic acid- iron solution had nearly no impact on the already formed complexes. The same effect was observed in another in *vitro study*, where ascorbic acid was added to a solution containing iron and tannic acid (Engle-Stone et al 2005). The results of the two studies indicate that EDTA and ascorbic acid can prevent the iron complexation by polyphenols, but they also indicate that only EDTA has the ability to weaken and destroy already formed iron polyphenol complexes. It should be taken into consideration that the study was conducted in a model *in vitro* system and results might only hint at compound interactions in the human host.

The discrepancy between the results of the above mentioned human studies and the *in vitro* study might be explained by the differences in pH. In the *in vitro* study pH was constantly 4.4, whereas pH in the human body rises from about 1 in the stomach to about 6 in the duodenum, where most of the iron is absorbed (Mccloy et al 1984). The strength of the EDTA- iron complex is determined by pH. It is strongest at pH 1 and constantly weakens with increasing pH (Lynch et al 1993). The results indicate that, in the duodenum at pH 6, the PP have a higher affinity to iron than EDTA, diminishing its effects.

#### *2.1.6.1.5 Reducing polyphenol levels in the diet*

As one of the major iron absorption inhibitors PP might have the potential to contribute to a low iron status, mainly in countries where people's diets are based on plants. However, until today no intervention studies investigated the influence of PP on iron status and there is only some evidence from observational studies that tea PP might have a negative impact on iron status (Gibson 1999, Merhav et al 1985). Nevertheless it has been clearly shown that PP strongly influence iron bioavailability. There are two possible approaches to counteract the reduction of iron bioavailability by PP, firstly to decrease their concentration and secondly to reduce their activity. Developing plants with low PP concentration is a strategy which has to be taken into consideration. Breeding beans with low PP concentration was suggested by Beebe and colleagues (2000). They discovered that the low PP concentration in white beans was not related to a lack of pigments, which would have caused difficulties in breeding coloured beans with low PP content. Moreover, they suggested that the variation in polyphenol levels within a single colour class could be higher than between the different colour classes, thus making a selection for low PP traits in the

different bean colour classes possible. No negative effect on plant resistance against pathogens seems to occur with reduction of tannins, an important factor to make this approach feasible. However, before implementing this strategy the effect of reducing PP concentration on the plant has to be investigated in more detail.

Another option to influence polyphenol concentration and activity is postharvest processing such as washing, drying, fermentation, germination, cooking, roasting and thermal treatment. Depending on the treatment, polyphenols are either lost (washing, soaking, dehulling) or eliminated/ oxidized (germination, fermentation, roasting). Soaking and cooking kidney beans decreased polyphenol contents by about 70 % with losses from both soaking and cooking being of similar magnitude and mainly due to leaching into water (Shimelis and Rakshit 2007).

Oxidation can, at least partly, be attributed to the enzyme polyphenol oxidase (PPO), which catalyzes the oxidation of the phenolic hydroxyl groups (Mayer 2006). It has been shown that flavanols are a good substrate for PPO and therefore are often reduced by this reaction (Amarowicz et al 2009). The enzyme is present in several fruits, vegetables and cereals, but can also be added from exogenous sources (Matuschek and Svanberg 2002). To activate the native enzyme, it might be important to disrupt samples since PP and enzyme are located in different cellular compartments. However, the decrease in PP concentration during germination can also be due to the complex formation of PP and seed proteins to hydrophobic compounds (Shimelis and Rakshit 2007). It can be summarized that the degree of PP elimination/loss strongly depends on the processing methods and on the PP subclasses present in the food. Research investigating the effect of postharvest processing and storage on PP content has been summarized in a recently published review (Amarowicz et al 2009).

## 2.2 Phytic acid

### 2.2.1 Occurrence

*Myo*-inositol-1,2,3,4,5,6- hexakisphosphate (IP6), also known as PA is the most abundant of all phosphorylated *myo*- inositol derivatives. Phytate, the salt of PA, is ubiquitous in eukaryotic species and is the major phosphorous and mineral storage form and comprises about 1-5 % of legumes, cereals, oil seeds, pollens, and nut. It is mainly located in the kernel, where it contains up to 75 % of the plants phosphorous, whereas other plant compartments, such as roots are rather low in phytate (Cheryan 1980, Raboy 2003). In most cereals, phytate is located in the aleurone layer, pericarp and the germ (Odell et al 1972), whereas highest concentrations in legumes can be found in the protein bodies of the endosperm or the cotyledon (Schlemmer et al 2009). Also other inositol phosphates can be found in plants such as inositol tetraphosphates and pentaphosphates, but to a far lesser concentration (about 15 %) than phytate (65 %- 80 %; Dorsch et al 2003). Phytate forms strong, mainly insoluble complexes with divalent and monovalent minerals such as iron, zinc, magnesium, copper, calcium and potassium. Thereby phytate provides the growing seedling and other plant compartments with essential minerals during ripening and maturation and is the crucial factor for good crop yields (Reddy and Sathe 2002). In contrast, the mineral binding properties of phytate are often regarded as one of the causes of mineral deficiencies in humans. But, aside from its properties as anti-nutrient, phytate exhibits some positive characteristics such as anticancer (Shamsuddin 2002) and antioxidative (Graf et al 1987) activities. Highest concentrations can be found in cereals and legumes and it is estimated that the daily consumption in the western world ranges from 0.3 g to 2.6 g per day. However, intake is much higher in the developing world, where people mainly consume diets based on plants. Detailed information about food sources, intake, processing and bioavailability is accessible through a recently published review (Schlemmer et al 2009).

### 2.2.2 Inhibition of mineral absorption

As already mentioned above, PA is highly negatively charged under physiological conditions and therefore has the ability to chelate positively charged minerals and

form insoluble complexes which are not available for humans. These complexes are soluble in the stomach under acidic conditions and precipitate at near neutral pH in the intestine (Schlemmer et al 2009).

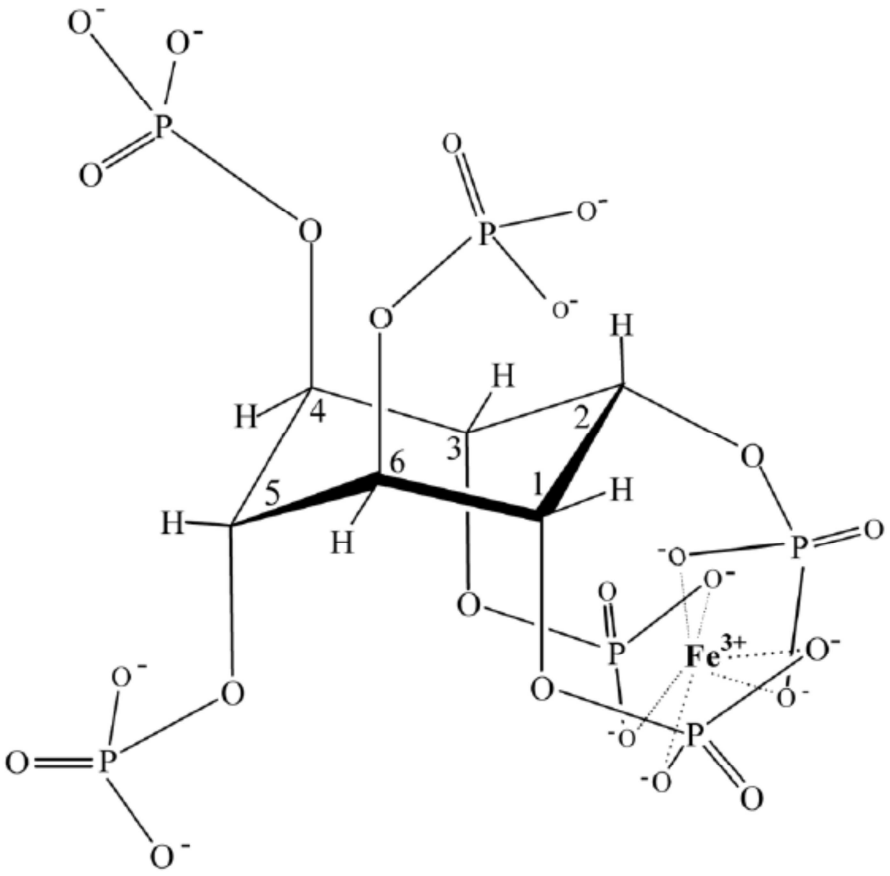


**Figure 10** Molecular structure of ferric-phytate; iron ($Fe^{3+}$-ion) is bound to phytic acid so that all six coordination sites of iron are occupied (Schlemmer et al 2009).

PA is in particular a strong inhibitor in iron absorption. The effect of PA on iron bioavailability has mainly been shown in iron isotope absorption single meal studies, where already small amounts of PA significantly reduced iron absorption (Hurrell et al 1992). However, single meal studies seem to be an reliable tool to measure the impact of PA on iron absorption since there is some evidence that no adaptation of iron absorption occurs during long term PA consumption (Brune et al 1989b). In this study the effect of high phytate bran on iron absorption of subjects with a high phytate intake over several years (vegetarians) was compared to a control group. No differences between groups were observed; adding bran to the test meal reduced iron absorption in both groups by more than 90 %.

The effect of PA on iron absorption is dose dependent, which first was observed in a study conducted by Hallberg (1989). They showed that 10 mg/ 100 g and 20 mg/100 g PA reduced iron absorption by 20 % and 40 %, respectively, whereas 100 mg/ 100 g PA reduced iron absorption by 60 %. This dose dependency was confirmed in another study, which, in addition, showed that the inhibition of PA on iron absorption can be overcome by ascorbic acid (Siegenberg et al 1991). But also other compounds such as EDTA have the ability to increase absorption from meals rich in PA (Troesch et al 2009). It is suggested in literature that in terms of inhibition the PA to iron molar ratio is more important than the total amount of PA. When meals are based on cereals or legumes complete PA degradation is suggested to improve iron bioavailability. If not possible, PA to iron molar ratio should be below 1:1 and preferably 0.4:1 (Hurrell 2004). In composite meals with meat or vegetables containing ascorbic acid a PA: iron molar ratio < 6:1 is proposed (Hurrell and Egli 2010, Tuntawiroon et al 1990). However, several isotope absorption studies have been conducted to investigate the impact of PA on other minerals. PA has also been shown to inhibit zinc (Navert et al 1985) and calcium (Weaver et al 1991) as well as magnesium (Bohn et al 2004) and manganese (Davidsson et al 1995) absorption. Two processes to overcome the inhibitory effect of PA on mineral absorption are suggested in literature. First, the mechanical removal of PA by processes such as extraction or milling. The later has been shown to remove up to 90 % of PA. The second is the enzymatic degradation of PA, which involves the activation of native phytase as well as the application of exogenous phytases (Hurrell 2004).

## 2.3 Inulin and Oligofructose

Inulin and oligofructose are fructans and belong to the general class of carbohydrates known as dietary fiber (Flamm et al 2001). Dietary fibers are non-starch polysaccharides which resist enzymatic digestion in the upper human gastrointestinal tract (Plaami 1997). They are divided into water soluble fibers (e.g. pectins, inulin or gums) and water insoluble fibers (e.g. cellulose, hemicellulose and lignin; Dikeman and Fahey 2006). Insoluble fibers are mainly cell wall components of wheat, most grain products and vegetables (Nair et al 2010). Soluble fibers are hydrophilic substances which form viscous colloidal dispersions or gels when hydrated (Ang and Crosby 2005). They can be found in fruits, legumes (e.g. beans) as well as in oat or barley (Nair et al 2010).

Dietary fiber have in common that they reach the colon intact, where they are either partly or totally fermented by the colonic microbiota or even remain unfermented (Flamm et al 2001). Fibers have different properties that lead to different physiological effects. Some dietary fibers delay gastric emptying, reduce postprandial blood glucose and blood cholesterol concentrations (Aro et al 1984, Jackson et al 1999, Medicin 2001), whereas those that are not fermented improve fecal bulk and ameliorate constipation (Medicin 2001, Schneeman 1997). On fermentation, dietary fiber is mainly metabolized to hydrogen, methane, carbon hydroxide and short chain fatty acids (SCFA; Roberfroid 1993). The ability to ferment depends on the chemical structure as well as on the solubility of the fiber (Nyman et al 1986), and soluble gums, pectins and inulin from fruits and vegetables are more readily fermented than cereal fibers such as cellulose and lignin (Schneeman 1997).

**Table 3** Constituents of dietary fiber according to the American Association of Cereal Chemists (AACC) (2001) and food sources (Belitz and Grosch 1992)

| | |
|---|---|
| ***Non-Starch Polysaccharides and Resistant Oligosaccharides*** | |
| Cellulose | Vegetables, woody plants, cereal brans |
| Hemicellulose | Cereal grains |
| Xylans | |
| *Arabinoxylans* | |
| *Arabinogalactans* | |
| Polyfructoses | Wheat, garlic, onion, chicory, artichoke |
| *Inulin* | |
| *Oligofructans* | |
| Galactooligosaccharides | Fruits, vegetables |
| Gums | Legumes, seaweed |
| Mucilages | All plants, mainly cactus and aloe vera |
| Pectins | Fruits, vegetables, legumes, potato |
| ***Analogous Carbohydrates*** | |
| Indigestible Dextrins | Typically produced by acid or thermal treatments of starch hydrolysates (e.g. from corn, potatoes) |
| *Resistant Maltodextrins* | |
| *Resistant Potato Dextrins* | |
| Synthesized Carbohydrate Compounds | |
| *Polydextrose* | Sweetener |
| *Methyl cellulose* | Emulsifier |
| *Hydroxypropylmethyl cellulose* | Thickening agent |
| Resistant starches | Bread, corn flakes, cooked beans |
| ***Lignin*** | Cereal brans, rice and legume hulls |
| ***Substances Associated with Non-Starch Polysaccharides and Lignin in plants*** | |
| Waxes | Plant cell walls |
| Phytate | Legumes, cereals |
| Cutin | Plant cell walls |
| Saponins | Soy beans, garlic, potato, tomato |
| Suberin | Roots and cork |
| Tannins | Tea, vegetables, fruits, legumes |

### 2.3.1 Properties of Inulin and Oligofructose

Inulin and oligofructose, also referred to as fructans, can be found in more than 36000 plant species, mainly serving the plant as energy source (Niness 1999). Inulin and oligofructose consist of ß (1-2)- linked fructose units build on a sucrose (dimer of glucose and fructose) precursor (Ritsema and Smeekens 2003), thus having usually one terminal glucose moiety (Figure 11).

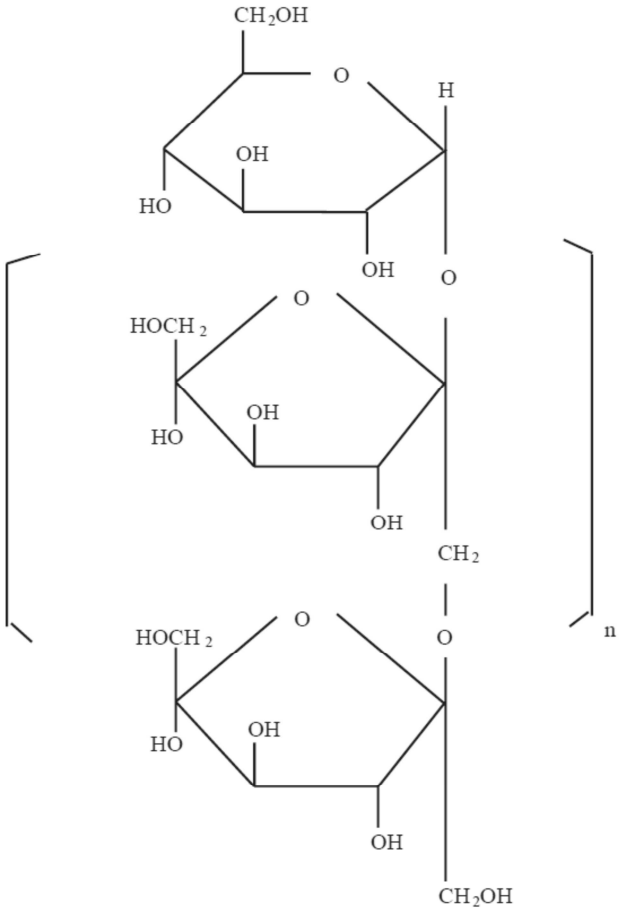


**Figure 11** Structure of inulin and oligofructose; n= 2- 60 (Tungland 2000)

The synthesis of fructans begins with the fructosyl transfer from one sucrose molecule to another, catalyzed by sucrose- sucrose fructosyltransferases. Following, chain elongation is mediated by a fructan- fructan- fructosyltransferase (Roberfroid and Delzenne 1998). The fructose chain differs in the degree of polymerization (up to 60). Oligofructose is obtained by partial hydrolyses of inulin; it is composed of the same fructose monomer but has a lower degree of polymerization (2-8; Bosscher et al 2006, Stevens et al 2001).

Both, Inulin and oligofructose are regarded as prebiotics since they selectively, unlike most dietary fiber, stimulate the growth and activity of specific bacteria, mainly bifidobacteria and lactobacilli with potential health promoting properties for the host (Roberfroid 2006, Steed et al 2008). They are naturally present in significant amounts in several vegetables such as garlic, artichoke, onion, asparagus, leek and wheat (1-4%).

**Table 4** Inulin concentrations of some selected foods (wet weight; Nair et al 2010)

| Source | Inulin (g/100 g) |
|---|---|
| Raw onion bulb (*Allium cepa*) | 1.1-7.5 |
| Jerusalem artichoke tuber (*Helianthus tuberosus*) | 16.0-20.0 |
| Chicory root (*Cichcorium intybus*) | 35.7-47.6 |
| Wheat (*Titicum spp.*) | 1.0-3.8 |
| Asparagus (*Asparagus officinalis*) | 2.0-3.0 |
| Garlic (*Allium sativum*) | 9.0-16.0 |
| Barley (*Hordeum vulgare*) | 0.5-1.0 |

Based on the consumption data, the daily intake of inulin in Europe ranges between 3.2 and 11.3 g mainly from wheat (2- 7.8g /d; VanLoo et al 1995). Also in the US the major food source is wheat (69 %), but mean intakes are lower and vary by gender and age groups ranging from 1.3 g for young children to 3.5 g for teenage boys and adult males (Moshfegh et al 1999). However, this might have changed recently since the food industry is adding considerable amounts into several products (Kolida and Gibson 2006).

### 2.3.2 The industrial application of inulin and oligofructose

The world market for prebiotics (food ingredients that stimulate the growth and/or activity of bacteria that are beneficial for human health: Gibson and Roberfroid 1995) and probiotics (microbes which have a beneficial effect on human health; FAO/WHO 2001) is rapidly growing. Already in 2008, 24 % of North American women and 13 % of North American men purchased pre/probiotic yoghurts and the consumption in Europe is equally strong with growing rates of 15 % to 20 % over the past 8 years

(Granato et al 2010). One of the major reasons for the enrichment of cereals, confectionary, biscuits, infant feeds, yoghurts, table spreads, bread, sauces and drinks with inulin and oligofructose are the potential health benefits (Kolida and Gibson 2006). Latest trends go towards the development of synbiotics, products containing health promoting bacteria as well as substances such as inulin which are promoting the growth and activity of these bacteria (Aragon-Alegro et al 2007). Most of the studies conducted with synbiotics focus on the prebiotic content as well as the activity/growth of bacteria and sensory quality of the product including formation and stabilization of foam, use of emulsifiers and stabilizers (Akin et al 2007, Aragon-Alegro et al 2007, Cardarelli et al 2008, Homayouni et al 2008).

Although results, investigating these factors in cheese, ice cream and chocolate mousse are very promising the effectiveness of synbiotics in terms of promoting human health has not been intensively studied. It is unclear if the health impact of pre- and probiotics is additive or synergistic. However, for industrial production also price, quality control, competitors, and economic factors play an important role in the development of such products (Granato et al 2010).

In addition fructans serve the food industry as additive to calorie reduced products. Either they are added as a sugar replacement since they only have 30 % less sweetness than sucrose or as a fat replacer. Their creamy fat-like texture enables the food industry to use them as a fat replacer in ice creams, dairy products and dressings (Stevens et al 2001). The estimated energy value is about 4.2 kJ/ g for inulin and approximately 6.3 kJ/ g for oligofructose (Roberfroid 1993) compared to 17 kJ/ g for sucrose and 38.9 kJ/ g for fat (Souci et al 1994). Several studies investigated the effect of inulin addition on dairy products. Workers found that yoghurt fortified with 1.3 % inulin showed no differences in viscosity and in acceptability when compared to control yoghurt (Dello Staffolo et al 2004). Others showed that inulin addition to fat reduced ice cream increased viscosity and improved the sensory properties (El-Nagar et al 2002, Schaller-Povolny and Smith 1999). The major inulin sources are *Cichcorium intybus* (chicory), *Dahlia pinuata Ca*V. (dahlia) and *Helianthus tuberosus* (Jerusalem artichoke), but the industrial manufactured inulin is nearly exclusively obtained from fresh chicory roots (Roberfroid 2006, Stevens et al 2001). Inulin concentration in chicory roots ranges from 15- 20 % fresh weight. It is mainly stored in the taproot as reserve carbohydrate (Niness 1999). Inulin is derived by extraction with hot water followed by a physical

separation step to yield products with purity up to 99 %. Oligofructose is then obtained by partial enzymatic hydrolysis (Roberfroid 2006).

### 2.3.3 The influence of inulin and oligofructose on gut microbiota

The mechanisms accounting for the composition of the human intestinal microflora are only poorly understood. But, it is known that the intestinal tract of a healthy, normal fetus is sterile and microbial colonization starts during the birth process (Mackie et al 1999). After birth infants become colonized with facultative anaerobes including *Escherichia Coli* and *Streptococci*. The exact composition of the bacteria is influenced by environmental factors as well as the mother's microflora and the type of birth delivery. But already during weaning the bacterial composition of the gut shifts and anaerobic species increase (Bettelheim et al 1974, Mackie et al 1999). The human large intestine houses several hundred species of bacteria with numbers of about $10^{11}$- $10^{12}$/ g. The majority are strict anaerobes such as Bacteroides, Bifidobacteria and Eubacteria, outnumbering aerobes and facultative species by a factor of 100- 1000 (Figure 12). Bacteroides and Bifidobacteria alone account for up to 55 % of total anaerobes, thus representing the largest number of bacteria in the human large intestine (Beaugerie and Petit 2004, Salminen et al 1998). In general, it can be said that bacteria which can most rapidly utilize the available substrates are greatest in numbers (Cummings and Macfarlane 1991).

Until some years ago identification of bacteria was based on classical cultivation techniques. Thus, leading only to a fractional knowledge of the bacterial diversity in the human gut since most bacterial species cannot be cultured (Amann et al 1995). New, modern techniques, based on sequence comparisons of nucleic acids (DNA, RNA) avoid the need of *in vitro* cultivation and allow the total characterization of gut microbiota (O'Hara and Shanahan 2006). Nevertheless limitation might occur due to the paucity of sequenced gene fragments, the use of fecal microbiota as substitute for gut microbiota and the variation associated with time, diet and health status (Eckburg et al 2005).

However, bacterial composition varies strongly between individuals, but is relatively stable within the same person. The growth of bacteria strongly depends on the supply with fermentable carbohydrates (Cummings and Macfarlane 1991) and it has been shown that the capability of different bacteria species to degrade carbohydrates strongly depends on the substrate structure (Leitch et al 2007).

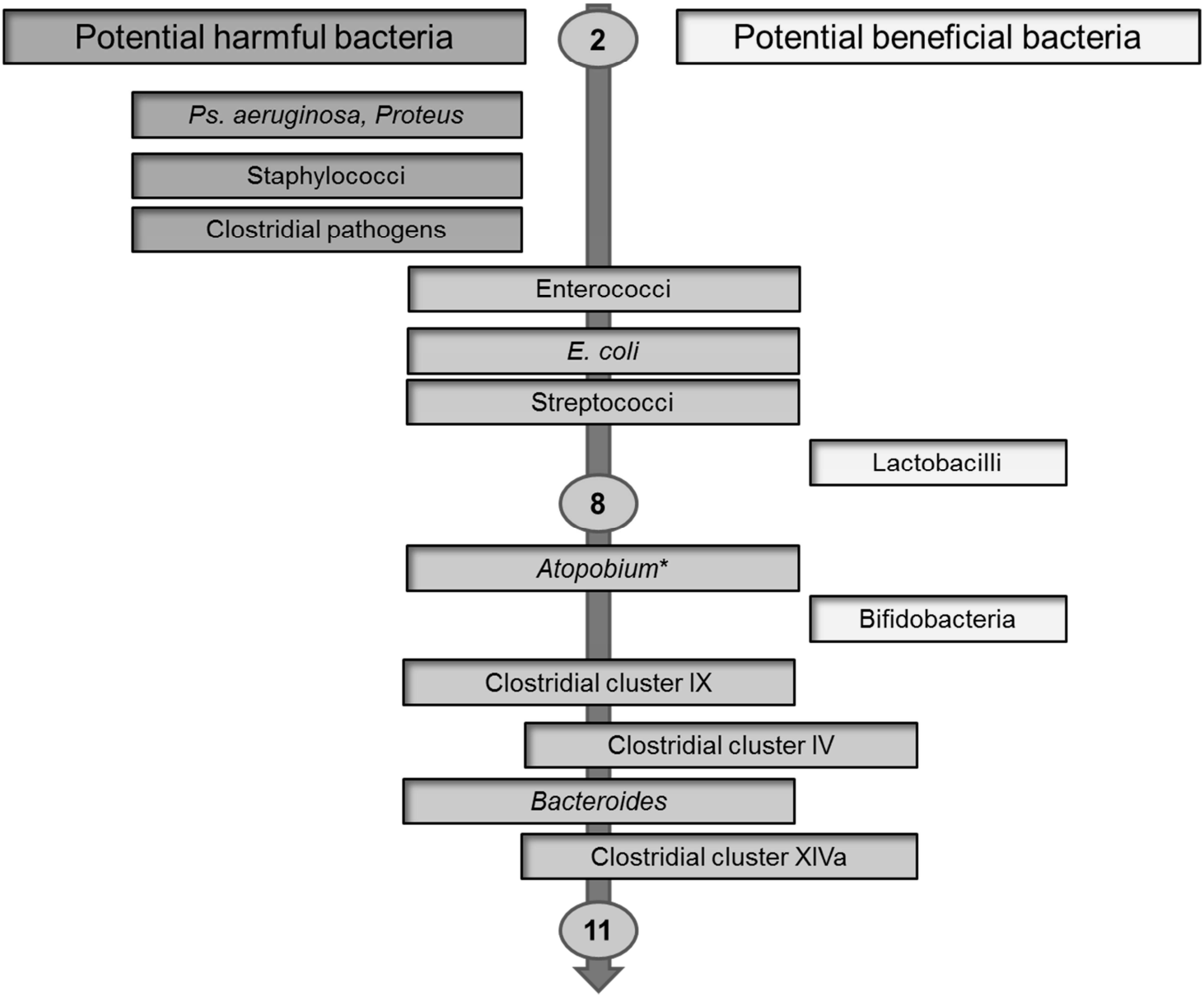


**Figure 12** Schematic presentation of the most abundant species and genera of the human colonic microbiota. The numbers at the arrow indicate approximate numbers of the different genera (Log 10 scale per gram of faeces). Diagram adapted from Meyer and Stasse-Wolthuis (2009)

Inulin and oligofructose significantly change the gut microflora by stimulating the growth of *Bifidobacteria* and *Lactobacilli*, which are both considered beneficial for human health. Latest studies showed that also other bacteria strains are able to respond to inulin and oligofructose (Duncan et al 2002, Duncan et al 2003). Among these bacteria are strains of the genera *Bacteroides*, *Streptococcus*, *Clostridium* and *E. Coli*, which can directly ferment and grow on inulin type fructans, but to a lesser extent (Mitsuoka et al 1987). However, the diverse response to inulin and oligofructose can also partially be explained by metabolic crossfeeding, which is the utilization of inulin breakdown products by other bacteria, supporting their growth (Belenguer et al 2006). It has to be taken into consideration that this, in turn, might lead to metabolic consequences which would not have been predicted from substrate preferences of isolated bacteria. This might, in rare cases, lead to a situation where Bifidobacteria are not the major group in the human gut responding to inulin.

The interaction between inulin and bifidobacteria is called "bifidogenic effect". This effect is relatively specific for fructans since bifidobacteria produce the enzyme β-fructosidase which is selective for ß (1-2) glycosidic bonds and enables them to enzymatically degrade inulin and oligofructose (Bouhnik et al 1996). Kaplan and Hutkins screened Bifidobacteria and lactic acid bacteria for their capability to degrade inulin and oligofructose. Seven out of eight *Bifidobacterium* strains and twelve out of sixteen *Lactobacillus* strains were able to ferment the substrates (Kaplan and Hutkins 2000). Studies trying to show the same effect with other dietary fibers failed (Gibson et al 1995). It has been shown that 5 g inulin per day for adults already positively modulates gut microbiota by increasing the level of Bifidobacteria (Bouhnik et al 2007). Thirty- nine healthy subjects were randomly allocated into 2 groups either receiving 2.5 g inulin or placebo twice a day. The workers observed a significant increase in Bifidobacteria in the inulin group, whereas pH, SCFA, fecal Enterobacteria and Lactobacilli concentration remained unchanged. They also detected a decrease in ß- glucuronidase, an enzyme which is mainly produced by potential pathogens such as *E. coli* and *Clostridium* bacteria (Cole et al 1985, Dabek et al 2008). The lower enzyme activity observed in the study might have indicated a reduction of pathogenic bacteria (Ling et al 1994), but results regarding the distribution of ß- glucuronidase activity between the different gut bacteria are conflicting (Cole et al 1985, McBain and Macfarlane 1998).

Other studies also found a decrease in potentially harmful bacteria such as Clostridia after administration of different fructans in adults (Gibson et al 1995) as well as in infants (Yap et al 2008).

To observe a significant increase in Bifidobacteria in infants already 1.25 g/day inulin has been shown to be sufficient (Yap et al 2008). An effect of fructans on Lactobacilli, which also belong to the probiotic bacteria, has only been detected in infants who had cancer (Zheng et al 2006), whereas mixtures of fructo- and galactooligosaccharides significantly increased the Lactobacilli concentration in healthy infants (Moro et al 2002).

However, the "bifidogenic effect" of fructans has been demonstrated in many studies feeding different fructans in different daily doses (Bouhnik et al 1996, Bouhnik et al 2007, Brunser et al 2006, Gibson et al 1995, Kleessen et al 1997). But several studies, most of them looking at the effect of oligofructose on infant gut microbiota (Bettler and Euler 2006, Euler et al 2005, Waligora-Dupriet et al 2007, Zheng et al 2006) and only some investigating the effect of inulin on adult bifidobacteria population (Calame et al 2009), failed to show an effect . It is assumed that the gut microflora modulating ability of fructans is independent of chain length (Meyer and Stasse-Wolthuis 2009) and daily dose. Roberfroid and colleagues (1998) compared data of several studies and concluded that the increase in bifidobacteria does not necessarily depend on the daily dose but rather on the initial number of bacteria in feces. They saw a stronger increase in subjects with lower initial bacteria counts but did not eliminate the possibility that a higher dose in the same subjects would lead to an even stronger increase.

### 2.3.4 Fermentation products of bacterial metabolism

Fermentation of non-digestible carbohydrates is an anaerobic process providing gastrointestinal bacteria with energy for growth and maintenance of cellular function. The major breakdown products of carbohydrates are SCFA such as acetate, butyrate, propionate, valerate and caprionate together with gases such as hydrogen, methane and carbon dioxide (Cummings et al 1987). The most prominent SCFAs, acetate, propionate and butyrate account for 85 to 95 % of total fecal SCFAs (Topping and Clifton 2001). Acetate concentration found in digesta is highest

followed by nearly equal amounts of propionate and butyrate (molar ratios approximately 3:1:1; Bergman 1990).

Further important products of carbohydrate fermentation are lactate, formate, ethanol, succinate, valerate and caproate which may be further fermented to SCFAs. Branched-chain fatty acids, such as isobutyrate, 2-methylbutyrate and isovalerate, are fermentation products of amino acids (Salminen et al 1998). About 90 % of the dietary protein and its breakdown products are degraded and absorbed, thus leaving 10 %, which reach the colon and are fermented by human gut bacteria (Elmadfa and Leitzmann 1998). Aside from branched-chain fatty acids the metabolism of proteins generates a series of potentially toxic substances such as ammonia, amines, phenols, thiols and indols (Macfarlane et al 1986a). Furthermore it has been shown that several potential pathogens are protein-fermenters, mainly growing in conditions which favor protein fermentation (Macfarlane et al 1986b). Not all bacteria present in the human gut have the ability to degrade polysaccharides. The most numerous carbohydrate utilizers are *Bacteroides* (Chassard et al 2007, Macy and Probst 1979), but also other fiber degrading species such as *Bifidobacterium, Lactobacillus, Ruminococcus, Eubacterium* and *Clostridium* are able to grow on carbohydrates. Bacteria which do not have the ability to utilize carbohydrates, can metabolize fragments produced by primary polysaccharide degraders (Figure 13; Gibson and Roberfroid 1995). E.g. hydrogen is utilized by oxidizers such as sulfate reducing and methanogenic bacteria (Gibson et al 1988). Several studies showed that intermediate and end products of bacterial carbohydrate fermentation strongly depend on the substrate. Starch fermentation, for example, is associated with a high concentration of butyrate, whereas pectin fermentation leads to a higher level of acetate (Englyst et al 1987).

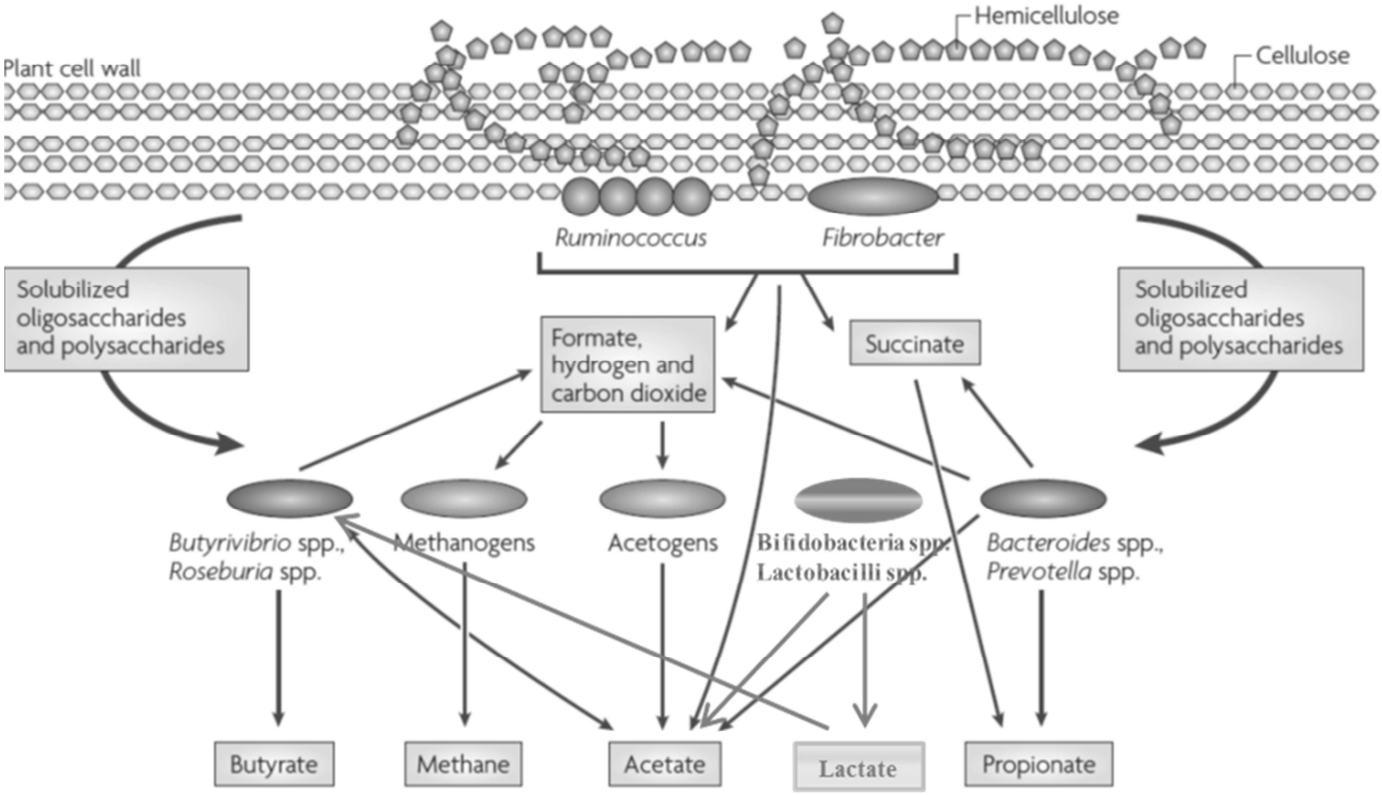


**Figure 13** The diagram is a schematic presentation of human gut bacteria and their breakdown products. It is illustrative and not intended to provide a complete description, as the number and diversity of primary degraders, polysaccharide utilizers and other functional groups within different gut communities is still emerging (Flint et al 2008)

More than 95 % of SCFA are produced and absorbed in the colon and final fecal output is only a small portion. In humans SCFAs contribute between 6 to 9 % to the daily energy requirements, which is little when compared to certain herbivorous animals where they make up to 85 % of the daily calorie intake (McNeil 1984).

Since most of the SCFA are absorbed, determination in humans is difficult. Some workers measured their concentration in the gut and in the portal venous blood after autopsy or during surgery (Cummings et al 1987, Dankert et al 1981, Peters et al 1992). Cummings and co-workers (1987) conducted a study in humans who had died suddenly. SCFA concentrations increased from 1 mmol/ kg in the jejunum over 13 mmol/ kg in the ileum to 131 mmol/ kg in the caecum. Concentrations then fell again in the colon from 123 mmol/ kg (ascending) over 117 mmol/ kg (transvers) to 80 mmol/ kg (descending). The obtained SCFA concentrations significantly correlated with pH. However, the mentioned approaches are obviously limited. Several workers measured SCFA concentrations directly in the stool or *in vitro* using

human fecal microbiota as fermenter. The measurements were usually done with gas chromatography (GC; Fernando et al 2008, Mills et al 1999) or high performance liquid chromatograph (Fernandes et al 2000, You et al 2001). But determination is difficult and recovery is often poor since SCFA are volatile and adsorb to metal and glass surfaces (Mills et al 1999).

### 2.3.5 Modulation of SCFA profile by inulin and oligofructose

The consumption of inulin and oligofructose significantly changes the SCFA composition in the human gut. As already described above inulin and oligofructose are mainly utilized by bifidobacteria and lactobacilli. Both bacteria species are predominantly acetate and lactate producers, although lactate is rarely found in human faeces. This is due to the fact that lactate is fully metabolized by other gut bacteria (Duncan et al 2004). Beards and colleagues (2010) looked at the influence of inulin fermentation on bacterial growth and SCFA production in faecal batch cultures. They observed a highly significant increase in Bifidobacteria and Lactobacilli as well as in the SCFAs acetate, propionate and butyrate. Also several other workers found an inulin related significant increase in acetate, butyrate and propionate production in rats (Demigne et al 2008, Levrat et al 1991).

However, these results are somewhat surprising at the first glance since Bifidobacteria do neither produce butyrate nor propionate, but the observed effect can be explained by metabolic crossfeeding. Belenguer and co-workers (2006) were able to show lactate crossfeeding between *Bifidobacterium adolescentis* and butyrate producing bacteria in vitro. Pure *Bifidobacteria* cultures were grown on fructans, resulting in lactate production ranging from 10 % to 30 % of total SCFA. The investigated butyrate producing bacteria were not able to grow on fructans but their number increased in cocultures with the Bifidobacteria strains. Thus, indicating that these bacteria were able to utilize Bifidobacteria breakdown products. Stable isotope techniques finally enabled the workers to monitor the carbon flow from lactate, via acetyl coenzyme A to butyrate and also acetate (Figure 14). Lactate conversion to butyrate, acetate and propionate was reported by Morrison and colleagues (2006). The same workers showed that, aside from lactate, acetate was metabolized and contributed to butyrate formation by human bacteria.

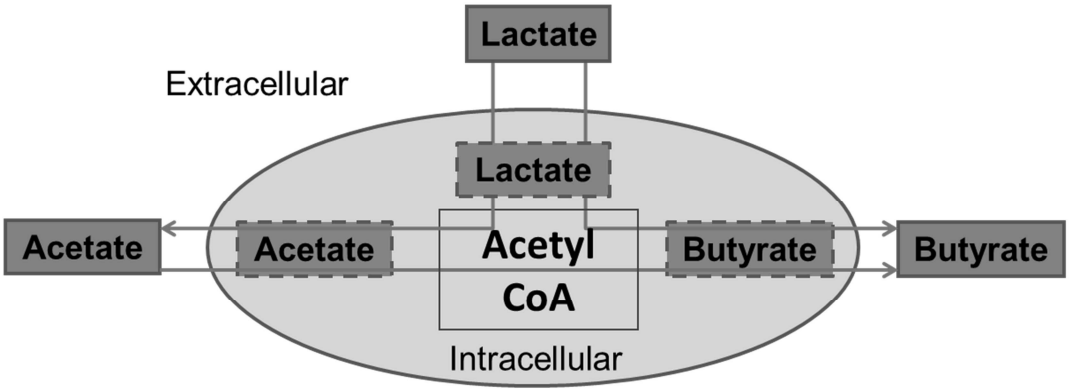


**Figure 14** Schematic representation of the model used for the $C_2$ flows between lactate, acetate and butyrate via acetyl CoA. Diagram adapted from Belenguer and colleagues (2006).

The same effect was observed in a further study using the two dominant butyrate producing species, *Faecalibacterium prausnitzii* and *Eubacterium rectale/ Roseburia spp.*, which account together for 5-10 % of total bacteria in faecal samples from healthy adults (Louis and Flint 2009).

In the published human studies, measurements were only done in faecal samples and to our knowledge neither inulin nor oligofructose at doses of between 4 and 40 g/ d produced any significant change in the concentration or molar ratios of fecal SCFAs (Alles et al 1996, Gibson et al 1995, Kleessen et al 1997). However, all these finding merely hint at the complexity of gut microbiota and their extensive metabolic interactions.

### 2.3.6 The impact of inulin on human health

As already described above, the consumption of fructans such as inulin and oligofructose stimulate the growth of Bifidobacteria and Lactobacilli and increases the concentration of SCFAs, mainly butyrate, in the human gut. Both, bacteria and SCFAs could have the capability to prevent intestinal infections and diseases in humans (Figure 15). Gibson and colleagues (1994) were the first showing that several Bifidobacteria strains inhibited the growth of pathogens in co-cultures by producing bacteriostatic substances. Until then it was believed that the inhibition of pathogens by Bifidobacteria was only related to the production of lactic and acetic acid as antibacterial agents (Fukuda et al 2011).

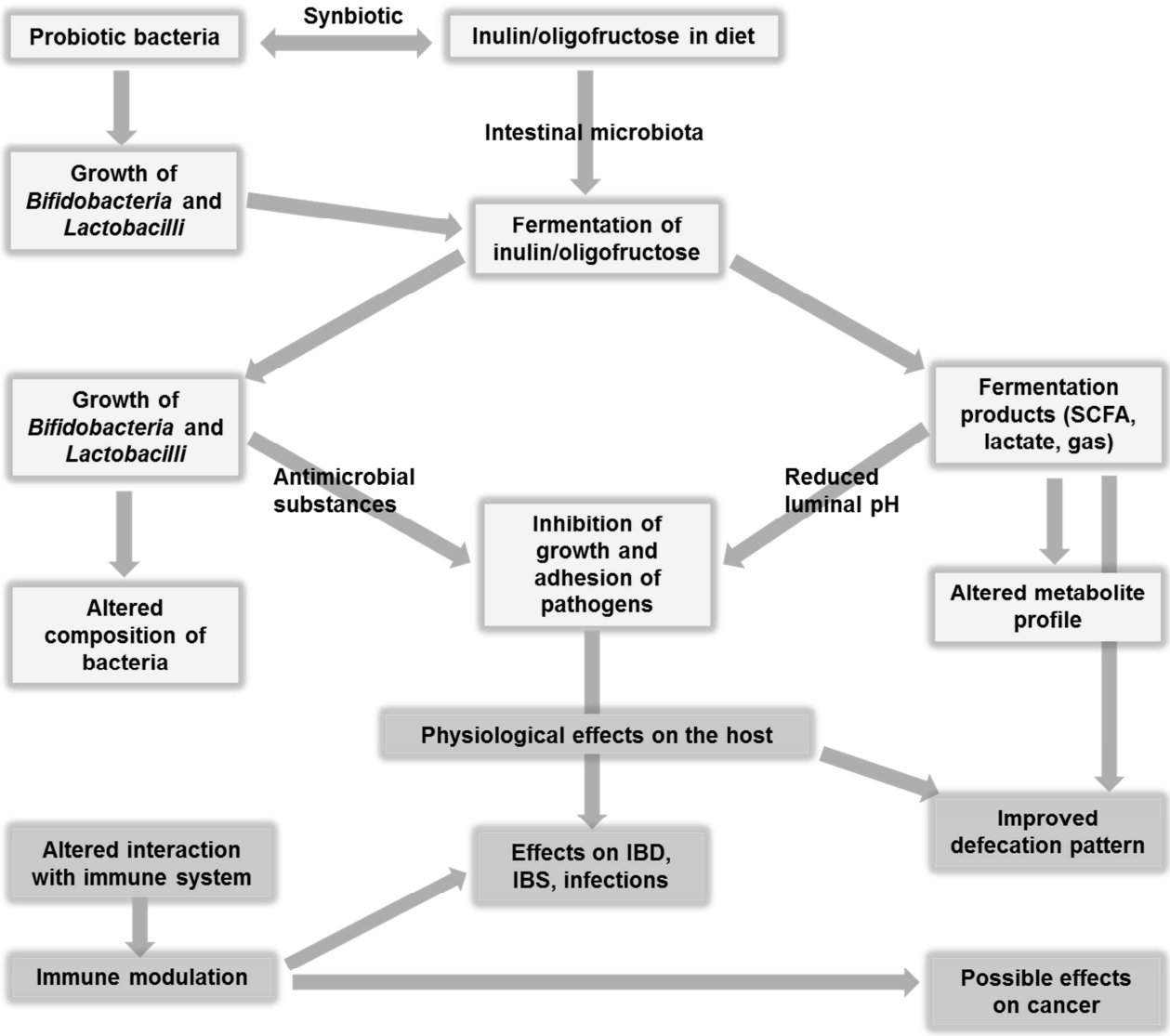


**Figure 15** Microbiota-dependent mechanisms of physiological effects induced by inulin/oligofructose or probiotic bacteria. (IBD= inflammatory bowel disease; IBS= irritable bowel syndrome). Adapted from Vos and colleagues (2007).

#### 2.3.6.1 Cancer prevention

Butyrate significantly reduces peroxide induced oxidative DNA damage (Rosignoli et al 2001), which might be one of the reasons for the lower prevalence of colorectal cancer in populations consuming high dietary fiber diets. It has been demonstrated by Beyer-Sehlmeyer and co-workers (2003) that butyrate as well as propionate suppresses the growth of tumor cells *in vitro*. But the observed growth inhibiting effect on the tumor cells was even greater from a mixture of SCFA and highest from fermentation products of different dietary fiber sources. The scientists concluded from

the results of the study that the activity from pure fiber sources such as inulin is only based on SCFA production during fermentation, whereas other dietary fibers have additional tumor growth inhibiting abilities through other plant ingredients such as PP.
The positive impact of fructans was also described in another study in 2009 were researchers observed an increased apoptosis and a reduced growth rate of tumor cells, when treated with fermentation fractions of inulin (Munjal et al 2009). Inulin and oligofructose have also shown their health protective and cancer preventive abilities in several animal studies (Buddington et al 2002, Klinder et al 2004, Poulsen et al 2002, Reddy et al 1997, Taper et al 1998). The prevalence of aberrant crypt foci (ACF), which is believed to be an indicator for potential carcinogenic effect, was significantly lower in mice receiving inulin and oligofructose than in mice fed with placebo. In the same study, mice fed with inulin and oligofructose had a much lower density of enteric pathogens (Candida albicans) compared to mice fed with cellulose. After infection with Listeria strains mortality rate in the mice control group was 28 %, whereas none of the mice fed inulin died (Buddington et al 2002). Paulsen and co-workers (2002) found a decrease in total ACF in rats fed long and short chain inulin, whereas long chain inulin was more efficient. Several other studies demonstrated the effectiveness of inulin against mucosal inflammation (Colitis) in rats (Cherbut et al 2003, Videla et al 2001) and humans (Welters et al 2002).

#### 2.3.6.2 Immune system modulation

Butyrate is physiologically relevant for the colonic epithelium, serving as a principal energy source and promoting cell proliferation (Roediger 1982, Wachtershauser and Stein 2000). Butyrate as well as acetate affect leucocytes and natural killer cell activity in the gut associated lymphoid tissue (GALT) and with it influence immune function (Inan et al 2000, Ishizaka et al 1993, Watzl et al 2005).
The effect of fructans alone or in combination with probiotic Bifidobacteria and Lactobacilli on the immune system is mainly concentrated on the GALT (Roller et al 2004, Watzl et al 2005). Oligofructose increased the production of IgA antibodies, which are important against viral pathogens, in infant mice (Nakamura et al 2004). Roller and colleagues (2004) were able to show that pre- and probiotics, individually and combined, positively influenced immune function. Oligofructose enriched inulin increased interleukin-10 production in Peyer's patches (PP) and activated T-

lymphocyte populations in the GALT, whereas sIgA was increased after the consumption of prebiotics and synbiotics. However, results indicated that pre- and probiotics acted via different mechanisms and that the effect of combined application is not additive.

In general it is assumed that the inulin and oligofructose attributed resistance against pathogens is mainly due to the change in gut microbiota towards potentially health promoting species and the associated increased production of SCFA (Roberfroid 2006).

#### 2.3.6.3 Lipid metabolism

The modulation of lipid metabolism and with it the reduction of atherosclerosis risk factors might be a further physiological effect of inulin-type fructans. Their daily consumption over a period of three weeks decreased blood lipid levels by reduced hepatic lipogenesis and also reduced plasma triacylglycerol concentrations in healthy subjects (Letexier et al 2003). Other workers detected a significant decrease in serum triglycerides and a trend toward reduction of serum cholesterol in hypercholesterolemic men who consumed 20 g inulin per day (Causey et al 2000). In a further randomized, double-blind, placebo-controlled study Brighenti and colleagues (1999) found that the daily consumption of 50 g of a rice-based cereal containing 18 % inulin significantly reduced plasma total cholesterol and triacylglycerols when compared to the control.

Possible mechanisms of inulin on lipid metabolism are widely discussed in literature but not clear. One mechanism might be the inhibition of cholesterol absorption during transit through the gut. Another might be the interruption of enterohepatic circulation of bile acids, leading to their excretion. Cholesterol is then used to synthesize new bile acids (Jenkins et al 1993). Third, SCFA, absorbed and transported via the portal blood to the liver, might have an inhibitory effect on hepatic cholesterol synthesis (Delzenne and Roberfroid 1994) and reduce, as in the case of propionate, plasma cholesterol levels (Trautwein et al 1998). However, results are controversy since several workers, feeding different amounts of inulin and oligofructose, found no significant improvement in lipid profiles (Davidson et al 1998, Luo et al 2000). A recent review which is discussing possible reasons and mechanisms concluded that

further *in vivo* studies with improved design are necessary to eliminate controversies (Ooi and Liong 2010).

#### 2.3.6.4 Possible negative health impacts

The results of studies investigating the health effects of inulin are not exclusively positive. Some workers observed no effect of inulin on Bifidobacteria population in the caecum of rats, but reported an increase in *Salmonella* colonization and translocation to extraintestinal sites (Ten Bruggencate et al 2004). This, oligofructose supported growth of salmonella, was also detected in mice (Petersen et al 2009). Furthermore a recently conducted *in vitro* study using the feces of children observed no inhibition of Salmonella growth by *Bifidobacterium thermophilum*. In addition the workers saw a stimulation of *Salmonella* growth when adding inulin at the end of the fermentation process (Zihler et al 2010).

Moreover, it has been shown that listeria infection was promoted by inulin in a pig model (Ebersbach et al 2010).

The fermentation of fructans, mainly fructans with a DP< 10 (fermented twice as fast as fructans with a DP> 10), leads to a strong increase in the concentration of luminal organic acids (Roberfroid et al 1998). This, in turn, might result in an irritation or even impairment of the mucosal barrier. However, changes in mucosal permeability occur at pH 5 and severe damage of the colonic mucosa at pH 4, both reversible (Argenzio and Meuten 1991). Furthermore inulin and oligofructose consumption might lead to symptomatic responses such as flatulence, rumbling, bloating, abdominal pain, abdominal cramps, diarrhea, nausea, increased stool frequency and changed stool consistency (Rumessen and Gudmand-Hoyer 1998). The upper tolerance level for fructans strongly depends on the individual. Bruhwyler and co-workers (2009) investigated the symptomatic response (flatulence, bloating, pain, etc.) of healthy subjects to different doses of inulin with varying chain lengths. They only detected mild symptom increase in the group consuming the highest concentration (20 g/ d) of inulin with an average DP of 10, when compared to placebo. Rumessen and Gudmand-Hoyer (1998) looked at the development of abdominal symptoms after consumption of different fructans and lactulose. They also detected only minor problems when subjects consumed less than 20 g inulin. The symptoms worsened at

an inulin concentration of 30 g where all subjects suffered from Borborygmia (stomach rumbling), 78 % from flatulence and 67 % from pain.
Apart from that inulin and oligofructose seem to have no direct negative effects on the human host. Several studies demonstrated that high levels of fructans have no negative impact on mortality, morbidity, organ toxicity, reproductive or developmental toxicity and carcinogenicity. Other scientists were able to show the absence of mutagenic and genotoxic potential of inulin in several *in vitro* studies (Carabin and Flamm 1999). To sum up, negative health effects of inulin and oligofructose are negligible at least where prevalence of salmonella infections are low, whereas health supporting and promoting properties are unmistakably strong.

### 2.3.7 The influence of inulin and oligofructose on mineral absorption

#### 2.3.7.1 Calcium

There is good evidence that inulin and oligofructose increase calcium absorption in humans. Calcium absorption can either be passive or active, whereas active absorption is vitamin D dependent and mainly takes place in the ileum. Passive transport occurs along the gastrointestinal tract by paracellular passive diffusion (Wasserman 2004) and is estimated to account for 8-23 % of total calcium absorption (McCormick 2002). Ninety to 95 % of human calcium absorption is located in the small intestine, whereas only about 5 %- 10 % occurs in the colon (Bargerlux et al 1989, Wasserman 2004).
Where the results of the enhancing effect of non-digestible carbohydrates on iron absorption are still controversy, the positive effect on calcium absorption (Abrams et al 2005, Coudray et al 1997, Griffin et al 2001, Holloway et al 2007, Tomita et al 2007, van den Heuvel et al 1999) and bone mineralization (Abrams et al 2005) in humans has been clearly demonstrated. The consumption of inulin or oligofructose over varying time periods in the different studies increased calcium absorption between 19 % (Griffin et al 2001) and 55 % (Coudray et al 1997). Most of the conducted studies however have been relatively short term. The only long term study looking at the effect of inulin on calcium absorption and bone mineralization has been done by Abrams and colleagues (2005). Young adolescents were supplemented with

8 g inulin or placebo/ day for 1 year. Calcium absorption was measured at baseline, after 8 weeks and at the end of the study by the use of stable isotopes. Bone mineralization was measured with dual- energy- X- ray absorptiometry. Calcium absorption in the treatment group was significantly increased after 8 weeks (30 %) and after 1 year (19 %) compared to the placebo group. Also whole- body bone mineral content and whole- body bone mineral density was significantly increased in the treatment group compared to the placebo group.

To our knowledge only one stable isotope study showed no effect of different non-digestible carbohydrates among them inulin and oligofructose on calcium absorption (van den Heuvel et al 1998), probably due to the poor study design. Calcium absorption was assessed by measuring the urinary excretion of calcium isotopes. Urine collection was stopped after 24 h, not taking into account the colonic calcium absorption.

It is suggested that the impact of fructans on calcium absorption might depend on their degree of polymerization (Coudray et al 2003). Griffin and colleagues (2001) conducted a crossover study, administering 8g/ d placebo, oligofructose and an oligofructose/inulin mixture for three weeks to 59 subjects. All phases were separated by a 2 week wash out period. Throughout the study subjects consumed about 1500 mg dietary calcium per day. Calcium absorption was measured at the end of each three week adaptation period using a dual isotope methodology. Isotope ratios were measured in a complete 48 h urine collection using thermal ionization magnetic sector mass spectrometry. The results showed that the inulin/oligofructose mixture significantly increased fractional calcium absorption from 32.3 %- 38.2 %, whereas the oligofructose alone had no significant impact.

However, it is commonly assumed that the effect of fructans on calcium absorption is restricted to the colon (Abrams et al 2007, Ohta et al 1994). Colonic calcium absorption accounts only for 5 % to 10 % of total absorption and studies reported an increase of up to 50 % of total calcium absorption after fructan administration. Thus, indicating that fructans exhibit an enormous potential to boost calcium absorption from the colon. Several mechanisms for increased calcium absorption due to inulin-type fructans are proposed in literature. Similar to iron, calcium absorption is assumed to increase with decreasing pH due to SCFA production (Ohta et al 1994). Apart from that it has been shown that SCFA directly stimulate calcium absorption in the colon independent of pH. The mechanism might be that protonated SCFA

diffuses into the cell and dissociates. The originated $H^+$ is exchanged for a $Ca^{++}$ from the colon and is than again able to protonate a SCFA to diffuse into the cell (Trinidad et al 1996). Another mechanism might be the butyrate induced cell proliferation and with it the gain in absorptive area (Lupton and Kurtz 1993, Scholz-Ahrens and Schrezenmeir 2002). Butyrate also stimulated the production of calbindin in the large intestine of rats (Ohta et al 1998a), which is involved in active calcium absorption.

#### 2.3.7.2 Iron

There is some evidence that non-digestible carbohydrates such as inulin and oligofructose have a beneficial effect on iron absorption (Yeung et al 2005). This impact has been observed exclusively in animals and studies trying to show the same effect in humans have so far failed.

As already mentioned above, inulin and oligofructose resist enzymatic digestion by the human host and reach the colon intact, where they are fermented by gut microbiota (Roberfroid 2006). Therefore the activity of fructans is mainly restricted to the colon and it is assumed that the interaction of fructans and iron must be situated in the colon as well. Consequently the colon must have the potential to significantly increase iron absorption, although major absorption takes place in the duodenum (Blachier et al 2007). This is because iron becomes insoluble and thus largely unabsorbable as it passes into the ileum and colon. However, in the pig, rat and mouse and presumably man, the ileum and colon also express the iron absorption proteins DMT1, ferroportin and hephaestin (Blachier et al 2007, Frazer et al 2001, Johnston et al 2006, Takeuchi et al 2005), although to a lower extent than the duodenum. Thus, if soluble iron was present in the ileum or colon some absorption would appear to be possible.

##### *2.3.7.2.1 Evidence for colonic iron absorption*

Early studies in humans indicated that orally administered iron is absorbed in 2 phases; a large fraction is absorbed in the first 2h and an additional amount in the following 22- 48 h (Wheby and Crosby 1963). While the second phase iron absorption could be the slow release of iron from mucosal cells, a significant proportion appears to be due to colonic absorption (Chernelc et al 1970) especially

as about 90 % of dietary iron is not absorbed in the duodenum and arrives in the colon. Ohkara and colleagues (1963) clearly demonstrated that humans can absorb iron from the colon when the provided iron is in the soluble ferrous form. Colonic absorption of iron infused into the colon as ferrous chloride was 7 %, or one third the absorption of orally administered ferrous chloride, both measured as erythrocyte incorporation. Ferric chloride infused into the colon however was only 0.5 % absorbed. In human subjects consuming a normal diet, these workers detected only small amounts of soluble ferrous iron in stools with a reported pH of 7-8.

There is further evidence for colonic iron absorption from a recently conducted study looking at iron isotope fractionation in a pig model. Natural iron is composed of four stable isotopes differing in their atomic mass. Although their abundance in nature is constant, small shifts may arise due to mass-sensitive processes where light and heavy isotopes are transferred at different rates. Circulating blood and the major iron absorption sites in the intestine have been shown to be enriched in light iron isotopes. Aside from duodenum and proximal jejunum, which are the main regions of iron absorption, the proximal colon was found to exhibit a high concentration in light iron isotopes, indicating its capability of iron uptake (Hotz et al 2011).

Low pH and also low iron status (Chernelc et al 1970, Panayotopoulos et al 1959) appear to favor colonic iron absorption in experimental animals. In radio iron studies with dogs, Chernelc and co-workers (1970) reported that colonic iron absorption was up regulated by iron deficiency to a far greater extent than duodenal iron absorption. After phlebotomy, absorption in the colon increased 6- fold to 15.7 %, whereas iron absorption from the duodenum only increased 3- fold to 30.8 %. They furthermore demonstrated the strong impact of pH on iron absorption. Iron absorption of iron directly injected into the colon at pH 2 was almost twice as high as the absorption of iron at pH 6.

Campos and colleagues (1996) confirmed that colonic iron absorption was up regulated in iron deficient rats and reported an increase in both passive and active iron absorption. Passive absorption was measured after the addition of 2,4 Dinitrophenol, an inhibitor of energy dependent absorption pathways. Passive colonic absorption of calcium, zinc and copper was also increased and these scientists speculated that the increase in passive absorption of all minerals was due to an increased permeability of the colonic membrane due to iron deficiency.

*2.3.7.2.2 Possible mechanisms of inulin on colonic iron absorption*

However, there are several possible mechanisms discussed in literature how inulin and oligofructose might influence colonic iron absorption. First, the fermentation products of inulin and oligofructose, mainly SCFA and lactic acid reduce pH in the colon and with it increase iron solubility and iron absorption. Ohta and colleagues (1995b) carried out a study looking at the effect of oligofructose on iron absorption in rats. Feeding oligofructose (50 g/ kilogram diet) to iron deficient anemic rats reduced pH by about 10-15 % and significantly increased hemoglobin concentration, hematocrit ratio and apparent iron absorption measured by assessing metabolic iron balance. Subsequently the same workers carried out further studies, in gastrectomized rats which usually develop a postgastrectomy anemia. In these studies they showed that oligofructose increased iron absorption and prevented anemia when fed with a non-heme iron containing diet (Ohta et al 1995). Oligofructose still stimulated iron absorption when fed with a heme iron containing diet, although the rats developed anemia (Ohta et al 1999).

In another study, three groups of anemic pigs were fed a corn soybean meal-based diet for five weeks without or with 2 % or 4 % inulin/oligofructose mixture. At endpoint hemoglobin concentration was 15 % higher in the 4 % inulin group when compared to the control group. No iron was added to the diet, showing that inulin increased the bioavailability of the intrinsically occurring iron. The inulin had no effect on pH of the digesta, but the workers found higher concentrations of soluble iron in the faeces of the treatment group. In addition, they found lower concentration of sulfide in the faeces of the treatment group (Yasuda et al 2006) which is generated by gut bacteria from sulfur amino acids and sulfate. Sulfide has been shown to complex iron and lead to its precipitation (Rickard 1995).

The second possible mechanism for an inulin increased iron absorption is that non-digestible carbohydrates or their metabolites increase the expression of iron regulatory genes. This was shown in a study conducted by Tako and colleagues (2008). They investigated the influence of inulin on the expression of DMT1, Dcybt, ferroportin, ferritin and transferrin receptor (TfR) genes in pigs. The pigs were allocated into two groups and fed with a standard maize-soya diet. The intervention group received in addition 4 % inulin. After 6 weeks the pigs were sacrificed and mRNA levels of the above mentioned genes in duodenum and colon measured.

Inulin significantly increased mRNA levels of DMT1, ferritin, Dcybt, ferroportin and TfR in the duodenum as well as ferritin, DMT1 and TfR in the colon, when compared to the control group, leading to the conclusion that inulin had a positive effect on iron absorption. It has also been shown that the impact of inulin on genes in the cecum and colon is stronger than on genes in the jejunum and duodenum, suggesting that inulin metabolites rather than inulin stimulate gene expression (Yasuda et al 2009). The supplementation of inulin did not only result in an up regulation of iron related genes, but also in a down regulation of inflammatory related genes. The latter might be a further explanation for the positive effect of inulin on iron absorption. In the presence of inflammation, iron absorption is decreased. This is mainly regulated by the activation of hepcidin transcription through inflammatory cytokines, especially interleukin 6 (Hentze et al 2010). Hepcidin is a hormone produced in the liver which controls plasma iron levels by regulating intestinal iron absorption, the release of recycled hemoglobin iron by macrophages and the movement of stored iron from hepatocytes. It binds ferroportin, an iron exporter on the surface of cells, and induces its internalization. Thus, leading to a decrease in plasma iron concentration (Nemeth et al 2004).

The third theory is that the stimulation of cell proliferation by SCFA leads to an increase of absorptive area in the colon which, in turn, might result in enhanced iron absorption (Yeung et al 2005). However, no studies are available to support this hypothesis and further investigations are necessary.

The fourth theory is based on an enhancement of iron absorption by an increased reduction of ferric to ferrous iron due to fermentation processes. It has been shown that the reduction of iron in presence of bacteria under anaerobic conditions in soil (100 %) is strongly increased when compared to processes in soil in absence of bacteria (3 %; Chen et al 2003). There is also some evidence from studies with rabbits that faecal fermentation products could reduce ferric to ferrous iron. Ohkawara and co-workers (1963) detected large amounts of ferrous iron in the faeces of rabbits and they reported some unpublished data indicating that the incubation of ferric chloride with the rabbit faeces led to a further increase in reduced iron.

A recently conducted intervention study by Sazawal and colleagues (2010) tested the effectiveness of milk fortified with *Bifidobacterium lactis* and oligofructose on reducing anemia and iron deficiency in children. Participants received either milk fortified with

*Bifidobacterium lactis* and 2.4 g oligofructose per day or control milk without any pre- or probiotics for a period of one year. Iron status (SF, TfR, and zinc protoporphyrin), anemia and growth were monitored. Hemoglobin as well as SF and TfR improved in both, treatment and control group compared to baseline. Consuming the fortified milk for one year reduced the risk of being iron deficient and anemic by 45 % compared to the control group. Workers found no differences in iron status parameters between the two study groups, but concluded however that long term treatment with pre- and probiotics may have a positive effect on iron deficiency anemia.

Human studies on inulin/oligofructose and iron absorption however have so far failed to show an effect. The first study, a metabolic balance study, was conducted in nine healthy men receiving up to 40 g inulin per day. After an adaptation period of three weeks faeces and urine was collected for 8 days between day 20 and day 28. The apparent iron absorption from the control meal was already very high at 21.8 % ± 12.3 % and did not further increase with the inulin diet (Coudray et al 1997). In the second study 12 healthy, non-anemic men with an average ferritin of 84 µg/l received a basal diet with 15 g/ d inulin, oligofructose, galactooligosaccharide or a diet with no added dietary fiber. The basal diet contained about 1 g calcium and 25 g dietary fiber. In the last week of the three week treatment period the subjects received iron isotopes, which they consumed together with the supplemented dietary fiber. The isotopes were added to 100 ml, vitamin C enriched orange juice with a total of 136 mg vitamin C. Two weeks after the last day of the treatment period isotope enrichment was measured in red blood cells. None of the non-digestible oligosaccharides had any effect on iron absorption, which ranged between 5 % and 6 % (van den Heuvel et al 1998). The negative results might be explained by presence of enhancers (vitamin C) and inhibitors (calcium; PA) which probably modified iron absorption in all test meals masking the effect of non-digestible oligosaccharides.

All animal studies, showing the positive effect of dietary fiber on iron absorption, were carried out with iron depleted mammals, whereas both human studies looked at the effect in iron sufficient men and as already described above, the results of Chernelc (1970) and Campos (1996) indicated that colonic iron absorption is only strongly elevated in iron deficient subjects. However, that inulin and oligofructose stimulate iron absorption was clearly demonstrated in animal models and that this effect was not observed in humans might have different reasons. One explanation might be the

poor study designs; another might be that the inulin related effect is restricted to the colon. Colonic iron absorption is, compared to duodenal absorption low and even a doubling might remain undetected. Further investigations are necessary to clarify the effect of inulin on iron absorption.

#### 2.3.7.3 Other minerals

Animal studies (Ohta et al 1995a, Ohta et al 1995b) as well as human studies (Coudray et al 2003, Tahiri et al 2001) showed a positive effect of fructans on magnesium absorption. It is suggested that magnesium absorption is mainly a passive diffusion process, only weakly controlled. But data is rare and mechanisms are less well understood than the mechanisms of iron and calcium absorption (Coudray et al 2002).

Beneficial effects of prebiotics have also been reported for copper and zinc, whereas studies were nearly exclusively conducted in animals (Coudray et al 2006, Delzenne et al 1995).

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# MANUSCRIPT 1

## Polyphenols and phytic acid contribute to the low iron bioavailability from common beans in young women

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**Abstract**

Low iron absorption from common beans might contribute to iron deficiency in countries where bean is a staple food. High levels of phytic acid (PA) and polyphenols (PP) inhibit iron absorption; however the effect of bean PP on iron absorption in humans has not been demonstrated and, with respect to variety selection, the relative importance of PP and PA is unclear. To evaluate the influence of bean PP relative to PA on iron absorption in humans, six stable iron isotope absorption studies were conducted in women (16 or 17 per study). Bean PP (20, 50, 200 mg) were added in studies 1-3 as red bean hulls to a bread meal. Studies 4– 6 investigated the influence on iron absorption of PP removal and dephytinization of whole red bean porridge, and PP removal from dephytinized porridge. Iron absorption was lowered by 14 % with 50 mg PP ($P< 0.05$); and by 45 % with 200 mg PP ($P< 0.001$). Mean iron absorption from whole bean porridge was 2.5 %. PP and PA removal increased absorption 2.6 fold ($P< 0.001$) and removal of PP from dephytinized porridge doubled absorption ($P< 0.001$). Between study comparisons indicated that dephytinization did not increase iron absorption in the presence of PP; but in their absence, absorption increased 3.4 fold ($P< 0.001$). These data suggest that in countries where beans are a staple food, PP and PA concentrations should be considered when selecting bean varieties for human consumption. Lowering only one inhibitor will have a modest influence on iron absorption.

**Introduction**

Iron deficiency (ID) is the most prevalent micronutrient deficiency worldwide, affecting mainly children under five and women of child bearing age living in the poorer communities of the developing world [1]. ID has a major negative impact on health and in pregnancy contributes to the risk of severe anemia, which is associated with higher maternal morbidity and mortality [2].

Iron fortification of foods or iron supplements have traditionally been the main intervention strategies used to combat ID, however they are less suitable for the more remote rural communities in the developing world where few processed foods are purchased or the health care infrastructure is poor. In such communities biofortification of staple foods could be a more cost effective and sustainable strategy. Biofortification is the process of increasing the level and/or bioavailability of essential nutrients in crops by traditional plant breeding or genetic engineering. Rice, wheat, maize, the common bean and cassava are the main targeted crops [3, 4]. Biofortified crops can potentially deliver iron, zinc and vitamin A to people in rural areas with limited access to commercial markets [5]. The common bean is a crucial grain legume since it is a major staple food in parts of Africa and Latin America [6] providing an important source of proteins and energy [6, 7]. It is also high in iron and zinc, and vitamins such as thiamin and folic acid [6, 8, 9].

The iron concentration of common beans (*Phaseolus vulgaris*) is generally higher than in cereal staples and has been reported to vary from 3.5 mg – 9 mg/100 g beans, depending on the genotype and appears to be relatively stable when grown under different environmental conditions [3, 10]. Selective plant breeding strategies have been reported to increase the iron concentration of common beans by 60-80 % [10]. Several human studies investigating iron bioavailability reported low iron absorption from beans in the range of 1-3 % [11-13]. Thus, for bean biofortification to have a positive impact on iron status, it would be beneficial to not only increase the iron concentration but also increase the iron bioavailability. Bean- based diets, like cereal- based diets, contain a considerable amount of PA; however, they additionally can be rich in phenolic compounds, mainly polymerized flavans [14-18]. Both PA and phenolic compounds can be potent iron absorption inhibitors, forming unabsorbable complexes in the gut lumen

[19, 20]. The inhibiting effect of PP on iron absorption has largely been demonstrated, but the capability of complex formation with iron in the intestine and thereby the reduction of iron uptake into the body depends on their structure [20-22]. The PP concentration in beans varies widely, depending on bean variety and color [16, 23] and it is likely that bean PP also inhibit iron absorption. However, this has never been tested. The molar ratio of PA to iron in beans ranges from 4:1 to 30:1 [23, 24], which would also be expected to markedly inhibit iron absorption [25, 26]. However, no data are available concerning the independent effects of PP and PA on iron absorption from foods containing considerable amounts of both inhibitors. The following six stable isotope iron absorption studies in adult human volunteers were designed to evaluate the relative importance of PA and PP on iron absorption from common beans so as to provide information that would enable plant breeders to develop beans with iron optimized for bioavailability.

## Methods

### *Participants*

Ninety-seven, apparently healthy, non-pregnant, non-lactating women aged between 18 and 45 y and below 60 kg were recruited from among the students of ETH and University of Zurich. Participants were randomly allocated to the 6 crossover studies, with 16 or 17 (study 6) participants per study (Table 1). Women with known metabolic, chronic and gastro-intestinal disease as well as woman on long-term medication (except oral contraceptives) were excluded from the studies. Intake of vitamin or mineral supplements was not allowed during and two weeks before the studies. No women were recruited who had donated blood or experienced substantial blood loss within 6 months of the beginning of the study. The experimental procedures were approved by the ethical committee of ETH Zurich and written informed consent was obtained from all study participants before the investigation began.

***Test meals***

In studies 1-3 (Table 1), the influence of different amounts of bean PP on human iron absorption was investigated. PP levels (20, 50 and 200 mg) were chosen, based on the concentration expected in 100 g low, middle and high PP bean, cooked and consumed without cooking water. Beans were first soaked and dehulled. The hulls, as the source of PP, were then steam-cooked before adding them to a non-inhibitory reference meal (RM) consisting of a bread roll (80 g) made from yeast fermented wheat flour, honey (7 g), and coconut fat (3 g). The amount of bean hulls added to the test meals provided 20, 50 and 200 mg PP (expressed in gallic acid equivalents; GAE), respectively. Each woman received a RM or a meal with added bean hulls on two consecutive days in random order. For dehulling, the beans were first cut in a circular manner with a sharp knife, so as to facilitate hull removal, and then soaked for 4.5 h at 4℃ and pH 5.5 to minimize PP losses. Before adding the hulls to the test meals, they were steamed for 15 min at 100 ℃ as a form of cooking. The bread rolls were prepared in batches by mixing 1 kg low extraction wheat flour with high-purity water (18 MΩ, 600 g), salt (10 g), sugar (32 g) and dried yeast (15 g). After fermentation for 5 h at room temperature, dough portions (80±1 g) were baked for 15 min at 200℃ and stored at -25℃ until the day of feeding.

In studies 4–6, the inhibitory effect of PP and PA in beans was investigated either individually or combined. In these studies, the test meals were in the form of sweetened, homogenized bean porridge. In study 4, the influence of bean PP on iron absorption in the presence of PA was evaluated by comparing iron absorption from beans with and without hulls; in study 5, the combined impact of PP and PA was investigated by comparing iron absorption from whole beans with dehulled, dephytinized beans and in study 6 the influence of PP on iron absorption in the absence of PA was evaluated by comparing dephytinized beans with dephytinized, dehulled beans.

**Table 1** Overview of iron absorption studies and test meals

| Study | Test meal A | Test meal B |
|---|---|---|
| 1 | RM[1] + bean hulls (20 mg PP) | RM |
| 2 | RM + bean hulls (50 mg PP) | RM |
| 3 | RM + bean hulls (200 mg PP) | RM |
| 4 | Whole bean meal | Dehulled bean meal |
| 5 | Whole bean meal | Dephytinized, dehulled bean meal |
| 6 | Dephytinized bean meal | Dephytinized, dehulled bean meal |

[1] bread roll (80 g), honey (7 g), and coconut fat (3 g)

For studies 4-6, the test meals were based on 60±1 g beans (Accession no.: SER 16; planted and harvested by CIAT, Columbia), either with or without bean hulls or with or without PA, soaked for 4.5 h at 4℃ and pH 5.5 and boiled in water for 40 min. After cooking, the beans were homogenized and 6.0 g sugar per test meal was added. For meal B in study 5, and meal A and B in study 6, 100 FTU Phytase (DSM FS Phytase 20.000 G; DSM, Delft, Netherlands) was added to the bean slurry after the homogenization and the slurry held at 55℃ for 60 min to allow complete PA degradation. One phytase unit (FTU) is defined as the amount of enzyme required to release 1 $\mu$mol of inorganic phosphorus per min from sodium phytate. The slurry was heated to 80℃ to inactivate phytase. The test meals were prepared in batches and stored frozen until the day of feeding. Four mg $^{58}FeSO_4$ or 4 mg $^{57}FeSO_4$ was added to each test meal in solution shortly before test meal administration. Exact amounts of added tracer were determined by weighing meals before and after addition of tracer solutions. High-purity water (18MΩ; 300 g) was served as a drink with each test meal.

***Study design***

Within each study, a randomized crossover design was used, in which each participant acted as their own control. In all studies, each woman received two different test meals labeled with either $^{57}Fe$ or $^{58}Fe$. On d 0, body weight and height was measured and the first blood sample was taken for iron status measurements. The following day (d 1), the first labeled meal was served between 0700 and 0900 after an overnight fast. On d 2, the second meal was administered in the same way. Women had to consume test meals including water completely in presence of the investigators. Participants did not eat or drink for 3 h after consuming the meal. Fourteen days after the second test meal (d 16), a second blood sample was taken after an overnight fast for Fe isotopic analysis. Fe absorption was calculated based on erythrocyte incorporation of Fe stable isotope labels 14 d after intake of labeled test meals (34).

The 97 participants in studies 1-6 were randomly assigned to groups of 16 or 17 (study 6) women each. Within each 2 day feeding period, the participants from all studies were randomly fed either test meal A or test meal B on d 1 and the other test meal on d 2 (see Table 1).

***Stable isotope labels***

Isotopically-labeled $^{58}FeSO_4$ and $^{57}FeSO_4$ was prepared from isotopically enriched elemental iron ($^{57}$Fe-metal: 95.3 % enriched; $^{58}$Fe-metal: 91.7 % enriched; both Chemgas, France) by dissolution in 0.1 mol/L sulfuric acid. The solutions were flushed with argon to keep the Fe in the +II oxidation state. Prepared iron tracer solutions were analyzed for iron isotopic composition and tracer iron concentration by reversed isotope dilution mass spectrometry using the experimental techniques outlined below.

***Analytical methods***

The total PP concentration in bean meals was measured with a modified Folin-Ciocalteau method as suggested by Singleton [27]. Iron, calcium and zinc in bean meals and bread rolls were analyzed by graphite furnace atomic absorption spectrophotometry (GF-AAS, AA240Z, Varian; Palo Alto, California) after freeze-drying. Beans and bread rolls were mineralized by microwave digestion (MLS ETHOSplus, MLS GmbH;

Leutkirch, Germany) using an $HNO_3/H_2O_2$ mixture. The PA concentration in bean meals and bread rolls was measured by a modification of the Makower method [28], in which iron was replaced by cerium in the precipitation step. Following the mineralization of food samples, inorganic phosphate was determined according to Van Veldhoven and Mannaerts [29] and converted into PA concentrations.

**Table 2** Total PP, PA, Iron, Zinc, Calcium, Magnesium and Ascorbic Acid (mg/meal) as fed in studies 1-6[1]

| Study | Test meal | PP | PA | Iron[2] | Calcium | Zinc | Magnesium |
|---|---|---|---|---|---|---|---|
| | | *GAE* | | | *mg* | | |
| 1 | A (RM + 20 mg PP) | [a]20 ± 0.3 | n.d. [4] | [a]4.7 ± 0.2 | [a,b]12.6 ± 0.3 | [a]0.6 ± 0.06 | n.a.[3] |
| | B (RM) | n.a.[3] | n.d.[4] | [a]4.6 ± 0.04 | [a]10.7 ± 0.5 | [a]0.6 ± 0.01 | n.a.[3] |
| 2 | A (RM + 50 mg PP) | [b]50 ± 0.9 | n.d. [4] | [a]4.7 ± 0.1 | [b]15.3 ± 0.2 | [a]0.6 ± 0.1 | n.a.[3] |
| | B (RM) | n.a.[3] | n.d.[4] | [a]4.6 ± 0.04 | [a]10.7 ± 0.5 | [a]0.6 ± 0.01 | n.a.[3] |
| 3 | A (RM + 200 mg PP) | [c]200 ± 3.4 | n.d. [4] | [a]4.8 ± 0.1 | [c]30.2 ± 0.5 | [a]0.6 ± 0.02 | n.a.[3] |
| | B (RM) | n.a.[3] | n.d.[4] | [a]4.6 ± 0.04 | [a]10.7 ± 0.5 | [a]0.6 ± 0.01 | n.a.[3] |
| 4 | A (Whole bean meal) | [d]187.4 ± 7.6 | [a]415 ± 10 | [b]6.1 ± 0.2 | [d]39.7 ± 3 | [b]1.1 ± 0.05 | [b]209.1 ± 7.5 |
| | B (Dehulled bean meal) | [a]27.9 ± 3.1 | [a]419 ± 11 | [b]6.2 ± 0.1 | [a,b]11.3 ± 0.3 | [c]0.9 ± 0.04 | [a]137.4 ± 5.8 |
| 5 | A (Whole bean meal) | [d]187.4 ± 7.6 | [a]415 ± 10 | [b]6.1 ± 0.2 | [d]39.7 ± 3 | [b]1.1 ± 0.05 | [b]209.1 ± 7.5 |
| | B (Dehulled, dephytinized bean meal) | [a]28.9 ± 2.2 | n.d. [4] | [b]6.0 ± 0.1 | [a,b]11.1 ± 0.4 | [c]0.9 ± 0.04 | [a]137.5 ± 1.1 |
| 6 | A (Dephytinized bean meal) | [d]177.9 ± 3.5 | n.d. [4] | [b]6.1 ± 0.2 | [d]37.7 ± 2.1 | [b]1.1 ± 0.1 | [b]205.3 ± 4.2 |
| | B (Dehulled, dephytinized bean meal) | [d]28.9 ± 2.2 | n.d. [4] | [b]6.0 ± 0.1 | [a,b]11.1 ± 0.4 | [c]0.9 ± 0.04 | [a]137.5 ± 1.1 |

[1]values are means ± SD, n= 3 independent analyses. Means in a column with superscripts without common letter differ, $P< 0.05$ (one-way ANOVA, Bonferroni)
[2] includes native iron and 4mg Fe added as $^{57}Fe$ or $^{58}Fe$
[3] n.a. = not analyzed
[4] n.d. =below limit of detection (< 8 mg/meal)

Ascorbic acid in honey was quantified by high performance liquid chromatography [30]. Wheat bran (PA assay) and milled beans (PP assay), stored under argon to avoid PP oxidation were analyzed together with each series of samples and were used as in-house quality control material to monitor reproducibility. Bovine liver (standard reference material 1577b; National Institute of Standards and Technology, Gaithersburg USA) was

measured together with each series of samples to monitor accuracy of the atomic absorption spectrophotometry method.

Whole blood samples were mineralized using an $HNO_3/H_2O_2$ mixture (7 mL/ 3 mL) and microwave digestion followed by separation of the sample iron from the blood matrix by anion-exchange chromatography and a solvent/solvent extraction step into diethyl ether (34). All isotopic analysis were performed by negative thermal ionization mass spectrometry (NTI-MS) using a magnetic sector field mass spectrometer (MAT 262; Finnigan MAT, Bremen, Germany) equipped with a multi-collector system for simultaneous ion beam detection [31, 32]. Venous blood samples were drawn in EDTA-treated tubes to determine iron status including hemoglobin (Hb) and serum ferritin (SF). Blood samples were divided into aliquots for the analysis of Hb and isotopic composition and plasma was separated, aliquoted and frozen for the later analysis of SF. Hb was measured with a Coulter Counter. SF and serum C-reactive protein (CRP) were measured on an IMMULITE® automatic system (DPC Bühlmann GmbH, Allschwil, CH).

***Calculation of Fe absorption***

The amounts of $^{57}Fe$ and $^{58}Fe$ isotopic labels in blood 14 d after administration of the test meals were calculated on the basis of the shift in iron isotope ratios and on the estimated amount of iron circulating in the body. Circulating iron was calculated based on the blood volume estimated from height and weight according to Brown et al. [33] and measured Hb concentration. The calculations were based on the principles of isotope dilution and took into account that iron isotopic labels were not monoisotopic [31]. For calculation of fractional absorption, 80% incorporation of the absorbed Fe into red blood cells was assumed.

***Statistical analysis***

Analyses were conducted with SPSS statistical software (SPSS 16.0; SPSS Inc.). Iron absorption values were converted to their logarithms for statistical analysis and reconverted for reporting. Iron absorption from different test meals within the same participant was compared by paired Student's t-test. A one-way ANOVA was used for comparisons between studies/participant groups. Following a post-hoc Bonferroni test

was used for multiple comparisons. Results are presented as means ± SD. Differences were considered significant at $P< 0.05$. To compare iron absorption between groups (reported in Figure 1), individual absorption values were adjusted to a serum ferritin concentration of 15 µg/L to consider the well-documented effect of iron status on iron absorption efficiency [34].The studies were powered to resolve a 30 % difference in iron absorption between test meals using each volunteer as her own control and to resolve a difference of 60% in absorption between groups.

**Results**

Four out of the 97 study participants had a hemoglobin concentration <120 g/L and 23 had a serum ferritin concentration <15 µg/L. Fifteen women showed a slightly elevated serum CRP concentration of >3 mg/L but none had a CRP concentration >10 mg/L. Their body mass index (BMI) was 20.5±1.4 kg/m$^2$ and their age was 22.7±2.9 y. Hb and SF concentrations were not different between any of the groups.

*Studies 1-3, influence of different amounts of bean PP on iron absorption*

RM fed with different amounts of bean hulls contained neither phytate nor ascorbic acid. Iron and zinc concentrations were constant over all test meals, whereas the calcium concentration increased with increasing amounts of bean hulls. PP concentration as planned was 20 mg GAE/meal in study 1, 50 mg GAE/meal in study 2 and 200 mg GAE/meal in study 3 (Table 2). The fractional iron absorption of participants consuming the RM in studies 1-3 (Table 3) did not differ. The lowest amount of PP (20 mg GAE), fed in study 1, did not affect iron absorption. Fifty mg of GAE from beans, fed in study 2, reduced mean iron absorption by 14 % ($P< 0.05$) whereas 200 mg GAE (study 3) decreased mean iron absorption by 45 % ($P< 0.001$).

*Studies 4-6, combined influence of PA and PP on iron absorption*

Dehulling of whole beans decreased the PP concentration by 85 % with a negligible effect on PA. Similarly, dephytinization of whole beans decreased the PA concentration to below analytical detection limits (8 mg/100 g) with little or no influence on PP concentration. The dehulled, dephytinized beans were low in both PP and PA.

Dephytinization had little influence on the iron, zinc, calcium and magnesium concentration of the whole beans, whereas dehulling resulted in relevant losses of zinc (18 %), magnesium (34 %), and calcium (72 %), but not iron (Table 2).

Iron absorption from the whole bean meal was similar in study 4 (2.6 %) and study 5 (2.4 %) in participants with similar iron status, as assessed by SF concentrations (Table 3). In study 4, removing the PP by dehulling was expected to increase absorption; however, iron absorption was decreased by 38 % ($P < 0.001$). In contrast, in study 5, removing both PA and PP increased iron absorption 2.6 fold ($P < 0.001$). In study 6, removing the hulls, and thus most of the PP from beans prior to dephytinization, doubled iron absorption (Table 3).

The influence of bean dephytinization was not measured directly in the same women in a single study but can be estimated by comparing iron absorption between studies. For between-study comparisons, iron absorption values were adjusted to a serum ferritin concentration of 15 µg/L as the cutoff level for iron deficiency [35] using experimentally derived algorithms [34], (Figure 1). After ferritin adjustment, iron absorption from the whole bean meal (meal A, Studies 4 and 5) was 4.9 %. Dephytinization in the presence of PP did not increase iron absorption; absorption from meal A in Study 6 was 5.7 %. Removing most of the PP in presence of PA did not significantly change iron absorption; absorption from meal B in Study 4 was 3.2%. Dephytinization after dehulling (meal B, Study 4), and removal of most of the PP increased iron absorption to 13.9 % (B meals in Studies 5 and 6, $P < 0.001$).

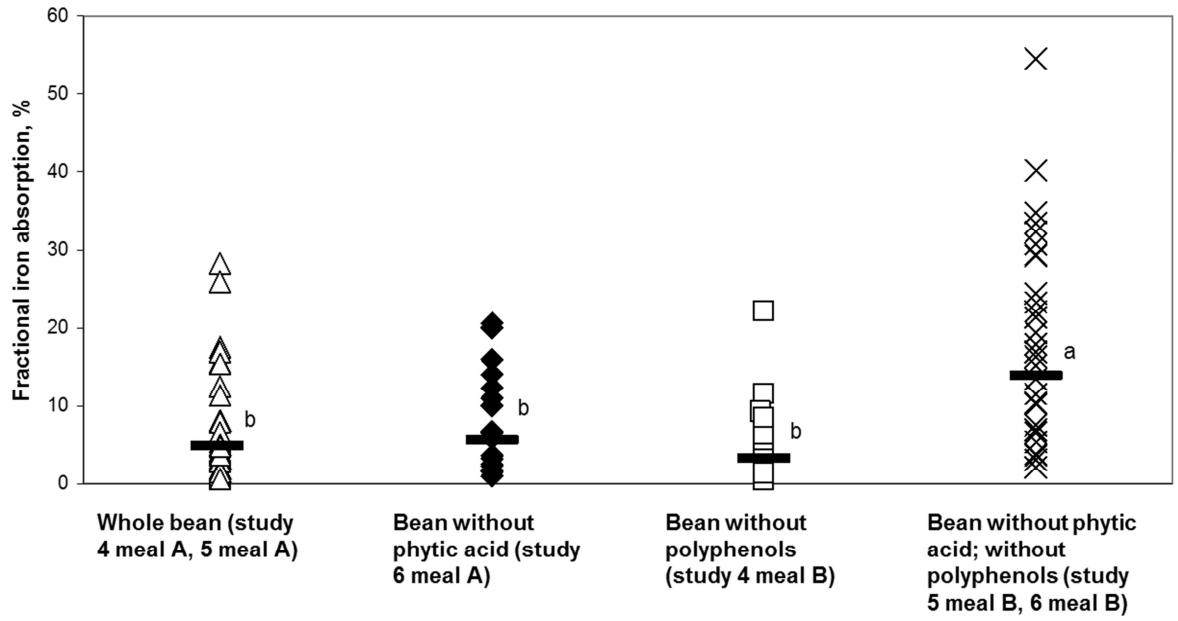


**Figure 1** Fractional iron absorption of women from test meals served in Studies 4-6 after normalization to a serum ferritin concentration of 15µg/L. Each symbol represents an individual participant. Geometric means are indicated by horizontal bars. Means without a common letter differ, $p < 0.05$.

## Discussion

Our results indicate that bean PP, as well as PA, contribute to low iron bioavailability from beans. We have shown that 50 mg and 200 mg GAE of bean PP (as quantified by the Folin-Ciocalteau method) decrease iron absorption by 14 % and 45 % respectively from a simple bread meal free of PA, whereas 20 mg GAE of bean PP had no effect. Bean PP would seem to be somewhat less inhibitory than the PP of common beverages, since Hurrell et al [20] reported that 200 mg GAE of PP from herb teas, black tea or red wine reduced iron absorption from a similar bread meal by 60-80 %, and 116 mg GAE of PP from cocoa reduced iron absorption by 70 %. The reasons for these differences are unclear, although it is known that PP from different foods can have different iron binding properties [21, 22].

Depending on their structure, they can form non-absorbable complexes with iron in the intestinal tract. Data suggests that PP with an *ortho*-dihydroxy (catechol) or trihydroxy-benzene group (galloyl) such as proanthocyanidins (catechol groups) and hydrolyseable tannins (galloyl groups) are the most potent iron absorption inhibitors [20, 21]. Common beans contain a wide range of flavanoids including proanthocyanidins, anthocyanins and

flavonols as well as phenolic acids [15, 36-38], and the PP concentration and profile are mainly determined by the seed color [10]. White beans contain phenolic acids, but anthocyanins and condensed tannins are not present [37-39], whereas red beans usually have the highest PP concentration [7, 10]. Variations in PP level within a single color class however can be higher than between the different color classes [10].

The three levels of PP used in our studies (20 mg GAE, 50 mg GAE, and 200 mg GAE) were estimated to represent the PP concentration of 100 g cooked beans of high (500-900 mg GAE/100 g), middle (150-350 mg GAE/100 g) and low PP (60-100 mg GAE/100 g) concentration [40]. PP amounts in our meals are based on 70 % loss of bean PP on cooking without consumption of the cooking water. Shimelis and Rakshit [41] reported that soaking and cooking kidney beans decreased PP concentrations by about 70% with losses from both soaking and cooking being of similar magnitude and mainly due to leaching into water. Soaking was tested with different pHs and temperatures and the optimal condition to minimize losses was found to be 4℃ and pH 5.5. For the interpretation of our results it should be emphasized, however, that we tested bean PP in a simple bread meal and that their inhibitory effect on iron absorption is likely to be less in more complex diets. On the other hand we increased the iron concentration of the meals by addition of isotopes and decreasing the PP to iron molar ratio would be expected to increase iron absorption.

**Table 3: Fractional iron absorption of women who consumed RM and bean test meals in studies 1-6[1, 2]**

| Study | n | Serum ferritin | Fractional iron absorption meal A | Fractional iron absorption meal B | Ratio[3] B/A |
|---|---|---|---|---|---|
| | | µg/L | % | % | |
| 1 | 16 | 17.8 (7.2- 43.9) | 13.9 (6.2; 31.3) | 14.2 (6.8; 29.8) | 1.02 |
| 2 | 16 | 18.0 (9.0- 36.3) | 20.2 (7.2; 56.7) | 17.3* (6.5; 46.4) | 0.86 |
| 3 | 16 | 18.1 (8.7- 37.7) | 14.3 (7.4; 27.9) | 7.9* (3.9; 15.7) | 0.55 |
| 4 | 16 | 29.7 (14.1- 62.4) | 2.6 (1; 6.7) | 1.6* (0.5; 5.1) | 0.62 |
| 5 | 16 | 29.3 (14.4- 59.6) | 2.4 (1; 5.7) | 8.7* (3.5; 21.5) | 3.63 |
| 6 | 17 | 24.4 (9.5- 62.4) | 3.5 ( 1.3; 9.5) | 7.1* ( 2.9; 17.3) | 2.0 |

[1] A meals contained 4 mg $^{57}$Fe; B meals contained 4 mg $^{58}$Fe
[2] Values are geometric means (range). *Different from meal A, $P < 0.05$ (paired t-test of log-transformed data)
[3] Absorption ratio meal B / meal A

Removing the PP from the dephytinized bean meal, by dehulling prior to dephytinization, confirmed their inhibitory effect in the absence of PA by doubling iron absorption (Study 6, Table 3). However, removal of PP from the whole bean meal (study 4) by dehulling unexpectedly led to a moderate but significant decrease in iron absorption (38 %; $p < 0.001$). Beans, and mainly bean hulls, contain various compounds, such as nondigestible carbohydrates [42] which might influence mineral absorption. Dehulling might have led to the loss of a compound positively influencing iron absorption. If such a compound exists it would need to be more active in the presence of PA (Study 4) than in its absence (Study 6). Our study design did not allow us to maintain a constant level of minerals over all test meals. A small amount of calcium and other minerals were removed with the hull (Table 2) but their removal would not be expected to decrease iron absorption [43]. However, to clearly interpret the results of study 4, further investigations are needed.

A negative effect of PA on iron absorption has been shown many times [19, 26]; as has the beneficial effect of removing PA from legumes such as soy [25]. As might be predicted from studies 1-3, removal of PA from whole bean containing some 180 mg PP did not increase iron absorption whereas dephytinization in the absence of PP increased iron absorption about 3.4 fold ($P< 0.001$). Estimated iron absorption from a whole bean meal in iron deficient women was 5 % (Figure 1), which is higher than expected from other studies and encouraging for bean biofortification [11-13].

From these studies, we conclude that both PA and PP inhibit iron absorption from beans and it seems that their inhibitory effect on iron absorption is not additive. It is likely, therefore, that modulating one without major changes in the other will have only modest effects on iron absorption and that the first priority of breeders should be to breed for a high iron concentration. The most common situation in relation to high bean diets is to have a relatively constant level of PA [44] but varying amounts of PP, depending on the bean variety. We have demonstrated the inhibitory effect of bean PP only in the absence of PA and, from our results, it is not possible to predict the influence of varying amounts of PP on iron absorption in the presence of PA. This will be the subject of future studies. The PA to iron molar ratio in the common bean is high (4-30: 1). Selective breeding for a lower PA level in high PP beans would not be recommended but selecting for lower PA varieties might improve iron absorption in communities consuming low or moderate PP beans, particularly if they were consumed within mixed diets with some enhancing foods [45, 46].

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# MANUSCRIPT 2

## The potential of the common bean (Phaseolus vulgaris) as a vehicle for iron biofortification

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**Abstract**

**Background** Biofortification of plant staples is a new, sustainable approach that could help combat iron deficiency (ID). Common beans (Phaseolous vulgaris) can be bred with higher iron content but are also rich in phytic acid (PA) and, if colored, in polyphenol (PP) compounds. Both are potent inhibitors of iron absorption.

**Aim** To better understand the potential of iron biofortified beans to combat ID.

**Method** Three iron absorption studies were carried out in Rwandese women (20 per study) of low iron status. Studies 1 and 2 compared iron absorption from high and low PP beans, similar in PA and iron, fed as a bean puree in a double meal design (study 1) or fed with rice and potatoes in a multiple meal design (study 2). Study 3 compared iron absorption from high and normal iron beans (9.1 or 5.2 mg Fe/100 g bean) with similar PP levels and a similar PA: iron molar ratio fed with potatoes or rice in multiple meals over 5 days. Iron absorption was measured as erythrocyte incorporation of stable iron isotope label at 14 d after meal consumption.

**Results** In study 1, mean fractional iron absorption from the high PP bean puree (3.4%) was 27% lower ($p<0.01$) than from low PP bean puree (4.7%). However when fed in multiple composite meals in study 2, there was no significant difference ($p>0.05$) in mean fractional iron absorption from the high PP bean (7.0%) and the low PP bean (7.4%). In study 3, mean fractional iron absorption from the high iron bean (3.8%) was 40% lower ($P<0.001$) than from the normal iron bean (6,3%) resulting in in equal amounts of iron absorbed ($p>0.05$).

**Conclusions** When high PA beans were combined with other meal components in multiple meals, their PP content did not have an additional negative influence to PA on fractional iron absorption. However, the quantity of iron absorbed from composite meals with high iron beans was no higher than with normal iron beans, indicating that efficacious iron biofortification may be difficult to achieve in beans rich in PA and PP.

## Introduction

Biofortification, the development of micronutrient dense crops by traditional plant breeding or by genetic engineering, is a promising new approach to combatting micronutrient deficiencies [1] and has the potential to be more sustainable and cost effective than food fortification or supplementation [2-4]. By distributing seeds which efficiently accumulate soil iron, the supply of iron to low-income households in rural areas with limited access to commercial markets could be increased [5]. In order to improve iron status however, the iron concentration and bioavailability as well as the consumption of the biofortified staple must provide an additional intake of bioavailable iron that makes a significant contribution to the gap between current iron intake and iron requirement. Additionally, so as to be accepted by the farmers, the biofortified crop must have a sufficiently high yield which is stable over different environments and climatic zones [6].

Current iron biofortification research programs focus on increasing iron content of staple crops such as wheat, maize, rice, beans and millet [1, 7-10]. The common bean is the major staple food for over 300 million people in Eastern Africa, Meso- and South America and is one of the most important legumes worldwide [11]. The iron concentration of beans varies between 3.5 mg and 9 mg/100 g, depending on the genotype, and is relatively stable when grown under different environmental conditions [12, 13]. Traditional plant breeding approaches have been reported to increase the iron concentration of some bean varieties by 60-80% [12, 14]. Beans however are high in phytic acid (PA), a potent inhibitor of iron absorption [15, 16]. Wheat and rice are also high in PA, but milling and polishing of the grains can substantially decrease its concentration and, with wheat flour, iron fortification compounds such as NaFeEDTA can be used to overcome the negative effect on iron absorption [17]. Cooked beans are neither milled nor fortified but are typically consumed whole without any separation process to decrease PA and the colored varieties are additionally rich in PP compounds which are also strong inhibitors of iron absorption [18].

Several human studies have reported low iron absorption from beans with published fractional iron absorption values ranging from 1.5- 2.6% [19-21]. While the PA content of beans is always high, the PP content varies considerably with the bean variety and color [22, 23], and it seems likely that only colored beans are sufficiently high in PP to additionally inhibit iron absorption. What is unknown however is

whether the inhibitory effects of PA and PP in beans are additive and to what extent the other components of a meal including beans facilitate iron absorption and thus dampen the inhibitory effect of PA and PP. Several researchers have reported a diminished effect of known inhibitors and enhancers when consumed with other foods in a composite meal [24-28].

The aim of the present studies was to better understand the potential of the common bean as a biofortification vehicle for iron. The three stable isotope studies were designed to evaluate whether the PP component of colored beans additionally inhibited iron absorption in the presence of high PA and to determine whether consuming beans with other meal components in composite meals decreases the inhibitory effect of beans on iron absorption. Finally the performance of biofortified high iron beans was tested by evaluating whether more iron is absorbed from composite meals containing high iron beans than from the same meals containing normal iron beans.

## Methods

### *Subjects*

The study subjects were selected from an initial screening of 230 women from the student and staff population of the National University of Rwanda gave a blood sample. Women with known metabolic, chronic and gastrointestinal diseases, on long-term medication, or who had donated blood or experienced significant blood loss within the 6 months prior to the study, were excluded. Sixty-one apparently healthy, non-pregnant, non-lactating women with marginal iron stores (serum ferritin < 25 µg/L), aged between 18 and 30 and below 65 kg body weight were recruited. The subjects were randomly allocated to one of three iron stable isotope absorption studies, each with 20 or 21 subjects. Intake of vitamin and mineral supplements was not allowed during the studies and for the two weeks before the studies. The experimental procedures were approved by the National Ethics Committee of Rwanda and the ethical committee of ETH Zurich. Written informed consent was obtained from all study subjects.

***Iron stable isotope studies***

Three separate iron absorption studies were undertaken (Table 1). Study 1 investigated the influence of bean PP on iron absorption from a high PA bean puree fed in a double meal design; study 2 investigated the influence of bean PP on iron absorption from a composite meal with beans, rice or potatoes fed in a multiple meal design; and study 3, also using a multiple meal design, investigated whether more iron was absorbed from a composite meal with rice or potato and high iron beans than normal iron beans. In study 1, the meals were fed on consecutive days and each subject received each of the test meals twice each day, either on day 1 or day 2. In studies 2 and 3, each of the test meals were fed twice per day on 5 consecutive days, either in week 1 or week 2.

**Table 1** Overview of iron absorption studies and test meals

| Study | Study type | Test meal A | Test meal B |
|---|---|---|---|
| 1[1] | Double meal | High PP bean | Low PP bean |
| 2[2] | Multiple composite meal | High PP bean + rice/potatoes | Low PP bean + rice/potatoes |
| 3[2] | Multiple composite meal | High iron bean + rice/ potatoes | Normal iron bean + rice/potatoes |

[1] test meals consisted of 75g beans. Each meal was fed twice on 1 of 2 consecutive days
[2] test meals consisted of 50g beans and 40 g rice or 170 g potatoes. Each meal was fed twice per day for 5 consecutive days during 1 of 2 consecutive weeks.

*Study 1*

The composition of the bean varieties tested in study 1 is shown in Table 2. The beans (FEB 226, red; and MIB 497, white; planted and harvested by CIAT, Colombia) were similar in iron and PA concentration but differed strongly in PP concentration. The test meals were fed as salted, homogenized bean porridge, containing 75±1 g beans (dry weight). The beans were first washed, soaked for 30 min at room temperature, boiled in water for 75 min, and homogenized. One g salt was added per test meal. The bean test meals were prepared in batches and stored frozen until the day of feeding. Two mg $^{58}$Fe or 2 mg $^{57}$Fe as a ferrous sulphate solution was added to each test meal shortly before administration. Two hundred g water was served with each test meal.

*Study 2*

The high polyphenol bean used in this study (SER 16, planted and harvested by ISAR, Rwanda) was a different red variety to that used in study 1 (FEB226) but was similarly high in PA and PP (Table 2). The low PP bean was the same as used in Study 1 (MIB 497). Iron absorption from typical African meals containing different bean varieties was investigated in two multiple meal studies (studies 2 and 3). In study 2, iron absorption from a meal containing a high PP bean (SER 16); was compared to iron absorption from a meal containing a low PP bean (MIB 497; planted and harvested by CIAT, Colombia). The test meal contained 50±1 g beans (dry weight) served either with 40 g rice (raw) or 170 g Irish potatoes. Beans were washed, soaked for 30 min at room temperature and boiled in water for 75 min. After cooking, beans were homogenized and 1g salt and 7 g soy oil per test meal were added. Beans were prepared in batches and stored frozen until the day of feeding. Polished rice was washed and potatoes were peeled, washed and cut into pieces before cooking. Soy oil (1.6 g) and 0.6 g salt was added to each portion. Rice and potatoes were prepared daily before feeding. The iron stable isotopes (0.4 mg $^{58}$Fe or 0.4 mg $^{57}$Fe) were added to each test meal in solution shortly before test meal administration. As the iron concentrations of SER 16 and MIB 497 differed by about 2mg iron/100g dry weight (Table 1), the iron concentration in the puree meals was equilibrated by adding 0.5 ml of a ferrous sulfate solution (1.5 mg Fe/ml) just prior to serving. As in study 1, 200 g water was served with each test meal.

*Study 3*

The composition of the high and normal iron beans is shown in Table 2. The high iron bean (MIB 465; planted and harvested by CIAT, Colombia) contained 9.1mg iron/100g compared to 5.2 mg/100g in the normal iron bean (SER 16). The same bean (SER 16) was fed as the high PP bean in Study 2. The PP content of MIB 465 and SER 16 were similarly high and, although the PA content of MIB 465 was considerably higher than SER 16 the PA:Fe molar ratios were similar (13:1). The bean meals in study 3 were prepared and fed with potatoes or rice as described for study 2.

***Feeding protocols***

The 61 subjects were assigned to studies 1, 2 or 3 in groups of 20 or 21 (study 3) women. A randomized crossover design was used and within each study each subject acted as her own control. On day 0, body weight and height were measured and the first blood sample was taken for iron status and inflammation measurements. The labeled meals were served in the morning between 0700 and 0900 after an overnight fast and a second meal three hours later. The subjects consumed the test meals (including water) completely in the presence of the investigators and were not allowed to eat or drink between the test meals and for three hours after the second meal.

In study 1, each woman received two test meals on two consecutive days each (d1 and d2) labeled with either $^{57}Fe$ or $^{58}Fe$. Fourteen days after the last test meal (d16), a second blood sample was taken after an overnight fast for Fe isotopic analysis. In studies 2 and 3, each woman received two series of 10 test meals. Test meals were served in the morning and for lunch from Monday to Friday for two consecutive weeks (d1-d5 and d8-d12) labeled with either $^{57}Fe$ or $^{58}Fe$. Fourteen days after the last test meal of week one (d 19) a second and fourteen days after the last test meal of week two (d26) a third blood sample were taken after an overnight fast for Fe isotopic analysis.

Fe absorption was calculated based on erythrocyte incorporation of Fe stable isotope labels 14 d after intake of the last labeled test meals [29]. Subjects of all studies were randomly allocated to either start with test meal A or test meal B.

***Stable isotope labels***

Isotopically-labeled $^{58}FeSO_4$ and $^{57}FeSO_4$ were prepared from isotopically enriched elemental iron ($^{57}$Fe-metal: 97.8 % enriched; $^{58}$Fe-metal: 99.4 % (study 1), 99.5 % (study 2 and 3) enriched; both Chemgas, France) by dissolution in 0.1 mol/L sulfuric acid. The solutions were flushed with argon to keep the Fe in the +II oxidation state. Iron tracer solutions were analyzed for iron isotopic composition and tracer iron concentration by reversed isotope dilution mass spectrometry as outlined below.

***Food analysis***

The total PP concentration in beans and bean meal components was measured with a modified Folin-Ciocalteau method as suggested by Singleton [30]. Iron in beans and bean meals was analyzed by graphite furnace atomic absorption spectrophotometry (GF-AAS, AA240Z, Varian; Palo Alto, California) after freeze-drying. Prior to iron measurements beans were milled. Freeze dried meal components and bean flour was then mineralized by microwave digestion (MLS ETHOSplus, MLS GmbH; Leutkirch, Germany) using an $HNO_3/H_2O_2$ mixture, which led to a complete oxidation of the samples, and thus to the destruction of the organic matrix. The PA concentration in bean meals and beans was measured by a modification of the Makower method [31], in which iron was replaced by cerium in the precipitation step. Following the mineralization of food samples, inorganic phosphate was determined according to Van Veldhoven and Mannaerts [32] and converted into PA concentrations. Wheat bran (PA assay) and milled beans (PP assay), stored under argon to avoid PP oxidation were analyzed together with each series of samples and were used as in-house quality control material to monitor reproducibility. Bovine liver (standard reference material 1577b; National Institute of Standards and Technology, Gaithersburg USA) was measured together with each series of samples to monitor accuracy of the iron estimation with atomic absorption spectrophotometry.

***Iron status measurements***

Venous blood samples were drawn in EDTA-treated tubes for the determination of hemoglobin (Hb), serum ferritin (SF) and C- reactive protein (CRP). Whole blood samples were divided into aliquots for the analysis of Hb and isotopic composition. Plasma was separated, aliquoted and frozen for the later analysis of SF. Hb was measured with a Coulter Counter. SF and serum C-reactive protein (CRP) were measured on an IMMULITE automatic system (DPC Bühlmann GmbH, Allschwil, CH). Hemolglobin was corrected for altitude (1500m) after the method of Dallman, which applies a 4% increase in hemoglobin concentration per 1 000 m of rise in altitude [30].

***Isotope analysis***

Whole blood samples were mineralized using an $HNO_3/H_2O_2$ mixture and microwave digestion followed by separation of the sample iron from the blood matrix by anion-exchange chromatography and a solvent/solvent extraction step into diethyl ether [29]. All isotopic analysis were performed by negative thermal ionization mass spectrometry (NTI-MS) using a magnetic sector field mass spectrometer (MAT 262; Finnigan MAT, Bremen, Germany) equipped with a multi-collector system for simultaneous ion beam detection [29, 33].

***Calculation of Fe absorption***

The amounts of $^{57}Fe$ and $^{58}Fe$ isotopic labels in blood 14 d after administration of the last test meals were calculated based on the shift in iron isotope ratios and on the estimated amount of iron circulating in the body. Circulating iron was calculated based on the blood volume estimated from height and weight according to Brown et al. [34] and measured Hb concentration. The calculations were based on the principles of isotope dilution and took into account that iron isotopic labels were not monoisotopic [29]. For calculation of fractional absorption, 80% incorporation of the absorbed Fe into red blood cells was assumed [34].

***Statistical analysis***

Analyses were conducted with SPSS statistical software (SPSS 16.0; SPSS Inc.) and Microsoft Office Excel 2003. Iron absorption values were converted to logarithms for statistical analysis and reconverted for reporting. Iron absorption from different test meals within the same participant was compared by paired Student's t-test. A one-way ANOVA was used for comparisons between studies and a post-hoc Bonferroni test was used for multiple comparisons. Results are presented as geom. means ± SD. Differences were considered significant at $P< 0.05$. The studies were powered to resolve a 30 % difference in iron absorption between test meals using each volunteer as her own control.

## Results

### *Subject characteristics*

All subjects had a low iron status (SF< 25 µg/L) (Table 3). Thirty nine (64%) of the 61 study participants had a SF concentration < 15 µg/L and 17 had a Hb concentration < 120 g/L. One woman showed a slightly elevated CRP concentration of >3 mg/L but none had a CRP concentration >10 mg/L. Mean body mass index (BMI) was 20.9± 2.2 kg/m$^2$. None of the parameters in Table 3 except BMI differed significantly.

**Table 3** Anthropometry and iron status of subjects[1]

| Variable | Study 1[3] | Study 2[4] | Study 3[3] |
|---|---|---|---|
| Weight[2] (kg) | 49.9[a] ± 4.3 | 52.5[a] ± 5.6 | 53.1[a] ± 6.7 |
| Height[2] (cm) | 159[a] ± 5 | 157[a] ± 6 | 157[a] ± 6 |
| BMI[2] (kg/m$^2$) | 19.6[a] ± 1.2 | 21.5[b] ± 2.4 | 21.5[b] ± 2.1 |
| Blood hemoglobin[2,5] (g/dL) | 12.7[a] ± 1.5 | 13.1[a] ± 1.2 | 12.6[a] ± 1.1 |
| CRP[2] (mg/L) | 0.7[a] ± 0.9 | 0.5[a] ± 0.7 | 0.7[a] ± 0.9 |
| SF[6](µg/L) | 14.4[a] (8.7; 24) | 9.3[a] (4.2; 20.6) | 10.3[a] (5.4; 19.6) |

[1] One-way ANOVA followed by a Bonferroni test was used for between study comparisons. Means in a column with superscripts without common letter differ, *P*< 0.05
[2] values are means ± SD (all such values)
[3] n= 20
[4] n= 21
[5] values corrected for altitude
[6] values are geometric means; range in parentheses

### *Bean and meal composition*

In study 1, PA and iron concentrations of FEB 226 and MIB497 did not differ significantly between the two bean varieties or between the cooked, homogenized bean test meals (Table 2). The PP concentration in the high PP bean was about 7 times higher than in the low PP bean (P<0.0001), although boiling in water reduced PP concentration of the high PP bean by about 50 % compared to only 4% in the low PP bean resulting in a 4 times higher PP concentration in the high PP bean meals (P<0.001) (Table 2).

The high PP bean fed in study 2 (SER 16) also had a PP concentration about 7-fold higher than in the low PP bean MIB 497 (P<0.001). As in study 1, the difference in PP concentration between beans was reduced to about 4 fold on cooking and

homogenization (P< 0.001). Iron concentration in the beans was significantly different (P< 0.001) but was adjusted in the test meals as described above. PA concentrations in both beans and bean meals were not significantly different (Table 2).

In study 3, iron concentration of the high iron bean (9.2mg/100g) was 75% higher than iron concentration of the normal iron bean (5.2 mg/100g) (P< 0.001) and final iron concentration in the test meals including the added isotopes differed by about 40 % (P<0.001) (Table 2). The beans had similar PP concentrations; cooking reduced PP in both beans by about 50%. PA concentrations in the beans however differed significantly (P< 0.001) with the high iron bean also having a high PA concentration resulting in a similar PA to iron molar ratio (9:1) in both bean meals.

Rice served in studies 2 and 3 had an iron concentration of 0.2 mg/100 g and a PA concentration of 10 mg/100 g dry weight. Iron concentration of Irish potatoes consumed in study 2 and 3 was 0.4 mg/100g as consumed.

**Table 2** Total PP, PA and iron in beans (mg/100g) and bean meals (mg/meal) as fed in studies 1-3[1]

| Study | Bean | PP | PA | Iron | Bean meal | PP | PA | Iron[2] |
|---|---|---|---|---|---|---|---|---|
| | | *mg/100g* | | | | *mg/meal* | | |
| 1 | High PP bean (FEB 226) | [a]653 ± 3 | [a]780 ± 3 | [a]6.6 ± 0.2 | Meal A | [a]259 ± 2 | [a,b]410 ± 10 | [a]6.9 ± 0.2 |
| | Low PP bean (MIB 497) | [b]89 ± 1 | [b]850 ± 11 | [b]7.3 ± 0.1 | Meal B | [b]65 ± 4 | [a]419 ± 11 | [a]7.3 ± 0.1 |
| 2 | High PP bean (SER 16) | [c]701 ± 8 | [b]854 ± 17 | [c]5.2 ± 0.1 | Meal A | [c]176 ± 3 | [b]393 ± 3 | [b]4.5 ± 0.2 |
| | Low PP bean (MIB 497) | [b]83 ± 2 | [b]867± 31 | [b]7.1 ± 0.1 | Meal B | [d]38 ± 5 | [a,b]402 ± 2 | [b]4.6 ± 0.2 |
| 3 | High Fe bean (MIB 465) | [c]728 ± 35 | [c]1392 ±11 | [d]9.1 ± 0.1 | Meal A | [d]163 ± 5 | [c]600 ± 3 | [c]5.7 ± 0.2 |
| | Low Fe bean (SER 16) | [c]700 ± 8 | [b]854 ±17 | [c]5.2 ± 0.1 | Meal B | [c,d]176 ± 9 | [b]393 ± 3 | [d]3.7± 0.1 |

[1]values are means ± SD, n= 3 independent analyses. Means in a column with superscripts without common letter differ, *P*< 0.05 (one-way ANOVA, Bonferroni)

[2] Study 1: includes native iron from beans and 2 mg Fe added as $^{57}Fe$ or $^{58}Fe$; Study 2 and 3: includes native iron from beans, rice and potatoes and 0.4 mg Fe added as $^{57}Fe$ or $^{58}Fe$; Study 2, meal A contains 0.75 mg iron added as ferrous sulphate to equilibrate the iron concentration

### *Iron absorption measurements*

Table 4 shows the fractional iron absorption and the total iron absorbed in µg per test meal. In study 1 mean fractional iron absorption from the high PP bean (3.4 %) was 27% lower than mean fractional iron absorption from the low PP bean (4.7%) ($p<0.01$) resulting in a significantly lower amount of absorbed iron. However this decrease in iron absorption with increased PP content was no longer found in study 2 when the beans were fed combined with rice or potatoes twice a day over 5 day as opposed to a single day in study 1. Fractional absorption values in study 2 were slightly higher at around 7%, and there were no differences in either fractional iron absorption or µg iron absorbed between the meals containing the high or the low PP beans. In study 3, mean fractional iron absorption from the normal iron bean was

6.3% and significantly higher (p< 0.001) than from the high iron bean (3.8%) resulting in no significant difference (p> 0.05) in the µg iron absorbed per meal. For between-study comparisons, iron absorption values were adjusted to a serum ferritin concentration of 15 µg/L as the cutoff level for iron deficiency [35] using experimentally derived algorithms [36]. After ferritin adjustment, iron absorption from SER 16 bean meals, fed within the multiple meal design of study 2 a and study 3 b did not differ significantly. Also iron absorption from the MIB 497 low PP bean meals (study 1 b and study 2 b) did not differ significantly.

**Table 4** Fractional iron absorption (%) and total absorbed iron (µg) per test meal

| Study | n | Bean meal | Total absorbed iron per meal[3,5] | P[4] | Fractional iron absorption[3,5] | Absorption Ratio | P[4] |
|---|---|---|---|---|---|---|---|
| | | | µg | | % | | |
| 1[1] | 19 | Meal A | [a]235 (115; 482) | <0.001 | [a]3.4 (1.6; 7.0) | 0.73 | <0.01 |
| | | Meal B | [a]341 (187; 622) | | [a,b]4.7 (2.6; 8.5) | | |
| 2[2] | 21 | Meal A | [a]317 (161; 622) | 0.164 | [b]7.0 (4; 14.1) | 0.95 | 0.61 |
| | | Meal B | [a]340 (174; 664) | | [b]7.4 (4; 15.2) | | |
| 3[2] | 21 | Meal A | [a]234 (85; 538) | 0.416 | [a,b]3.8 (1.5; 9.4) | 0.60 | <0.001 |
| | | Meal B | [a]225 (111; 493) | | [a,b]6.3 (3; 13.34) | | |

[1]all meals contained 2 mg $Fe^{57}$ or 2 mg $Fe^{58}$

[2]all meals contained 0.4 mg $Fe^{57}$ or 0.4 mg $Fe^{58}$

[3]values are geometric means; range in parentheses

[4]paired Student's t-test was used to compare differences in total iron and fractional iron absorption on logarithmically transformed data within each study

[5]One-way ANOVA followed by a Bonferroni test was used for between study comparisons. Means in a column with superscripts without common letter differ, *P*< 0.05

## Discussion

This study has two major findings. The first is that the inhibitory nature of bean PP on iron absorption [18] did not further increase the inhibitory effect of beans per se when the beans were consumed in composite meals with potatoes or rice over a period of 5 days. This would indicate that white beans and colored beans would provide similar amounts of bioavailable iron when consumed in traditional meals. The low iron absorption from white beans and white bean meals can be explained by the high

phytate content of beans [23, 37] and by the inhibitory nature of legume proteins [38, 39].

However, when the beans were consumed alone (Study 1, Table 4), over two meals on a single day, iron absorption from the high PP colored bean was 27% lower than for the white bean. In this study, in which the meals contained only beans and were fed twice on a single day, the inhibitory effect of the PP was additional to the inhibitory effect of phytate and legume proteins. Finding no additional inhibition of bean PP in the composite meals (Study 2) is perhaps not surprising as other food components in a meal has been reported to decrease the inhibitory effect of PP [28]. It is possible that perhaps other food components in potatoes or rice facilitated iron absorption from the bean meals due to their reducing or chelating effects, or simply diluted the effect of the inhibitors. In addition, single meal studies have been reported to exaggerate the inhibitory effect of food components when compared to multiple meal studies [36, 40] and the different study duration could offer a further explanation for the apparent discrepancy between studies 1 and 2. It should be noted that different high PP bean varieties were fed in study 1 and 2 and it is possible that different individual PP compounds in these 2 varieties had different inhibitory potential.

The second finding, that the high iron bean did not provide a greater amount of absorbed iron when fed in multiple composite meals over 5 days, questions the usefulness of beans as a vehicle for biofortified iron. This concern was already raised by Donangelo and King [20] after similar results to our own. They compared iron absorption from two bean varieties, one being 65 % higher in iron, lower in tannin concentration and in PA-to-iron molar ratio (20:1 vs. 30:1). Iron absorption from both beans was low and no difference in the total amount of iron absorbed from the two bean types was detected.

Beans and other cereal staples are high in phytate. The inhibitory effect of phytate in cereals can partially overcome by milling or polishing and, where cereal flours are centrally processed, they can be fortified with iron compounds such as NaFeEDTA which overcome phytate inhibition. Bean phytate is within the protein bodies of the cotyledon [37] and is not possible to remove by milling and, because beans are not centrally processed, providing iron fortified bean flour is not an option. Biofortification is thus the best solution.

Our results however indicate that in the presence of high levels of phytate, polyphenols and maybe also inhibitory proteins, high iron beans may not provide additionally useful amounts of bioavailable iron. In our studies, the beans were fed twice a day over a 5 day period so provide stronger information than single meal studies however it is possible that over month or years they would still help maintain or improve iron status. A recent evaluation of national wheat flour fortification programs [41] has recommended an additional 6 mg fortification iron per day as ferrous sulphate so as to usefully improve iron status. While doubling the iron content of beans and consuming 100 g or more of beans per day would approach this value, the more inhibitory nature of beans as compared to low extraction wheat flour may make it more difficult to absorb sufficient quantities of biofortified iron. The mean fractional iron absorption values of around 7% from composite bean meals by the women of low iron status however offer some encouragement.

It was somewhat surprising that more iron was not absorbed from the high iron bean by the subjects of low iron status. If more iron was available for absorption from the high iron bean, it would be expected that more iron would be absorbed. However, around 200 µg iron per meal was absorbed irrespective of the bean. Previous studies have reported that fractional iron absorption goes down as the quantity of iron ingested increases but that more iron is absorbed. Cook et al. [42] added 1, 3 and 5 mg labeled ferrous sulfate to a bread roll meal. Fractional iron absorption decreased, but total amount of iron absorbed increased. Additionally it is assumed that increasing the amount of fortification iron increases its ability to improve iron status [41].

The most logical explanation for our finding is the strong inhibitory nature of phytate in the high iron bean resulted in a similar amount of bioavailable iron being released during digestion in the gastrointestinal tract as is released from the normal iron bean. While phytate level usually increases with iron level, the phytate to iron molar ratio usually decreases (Hoppler et al unpublished). In the high iron bean fed in our study, the phytate level was exceptionally high and the phytate to Fe molar ratio was similar to the normal iron bean. Because of this, we would have predicted a similar fractional iron absorption in the normal and high iron beans, however if iron and phytate are in different compartments or released into the stomach at different times, it is possible that the higher phytate content of the high iron bean had a greater inhibitory effect on iron absorption. While iron speciation in beans has not been extensively studied,

recent results from our laboratory (Hoppler et al. unpublished) with beans of varying iron content indicate that ferritin iron is at a similar concentration in all beans and that bean iron is increased by an increase in non-ferritin iron. The non-ferritin iron is possibly linked to phytic acid and may behave differently to ferritin iron.

The way forward would seem to be to select high iron beans with low phytate and polyphenol content. Recently an lpa mutation (lpa 280-10) was isolated in the common bean [43] which has been mapped on chromosome 1 and characterized at biochemical and molecular level, indicating it is caused by the loss of function of a vacuolar transporter [44]. This mutant line showed a 90 % reduction of PA and much higher concentration in free or weakly bound iron. Agronomic trials performed over 2 growing seasons revealed that the mutant germination rate, seed yield and other relevant agronomic characters are similar to those of its parents, indicating this is the first lpa mutation devoid of visible negative effects in plants, pods and seeds [43]. The mutation, originally in a black-coated bean, could be introduced into white-coated beans with very low level of PP so as to maximize iron absorption.

In conclusion, these studies indicate that when beans are consumed as part of composite, traditional meals, the inhibitory nature of bean PP does not further inhibit iron absorption. They also suggest that increasing the bioavailable iron content in high phytate, high PP beans may prove difficult and that the way forward for iron biofortified beans is to select for high iron, low phytate and low PP content.

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# MANUSCRIPT 3

## Inulin modifies gut microbiota, fecal lactate concentration and fecal pH but does not influence iron absorption in women with low iron status

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**Abstract**

Bioavailability of non-heme iron is strongly influenced by the concentration of inhibitors and enhancers in the diet. The β (1-2) fructans, inulin and oligofructose have been shown to beneficially influence calcium absorption in humans through colonic uptake and an increased colonic iron absorption has been demonstrated in rats and pigs but not confirmed in humans.

A double blind randomized crossover intervention study was conducted in women (n=32) with low iron status to evaluate the influence of inulin on iron absorption by means of stable iron isotope techniques. Bifidobacteria, total bacteria, short chain fatty acids (SCFA) and stool pH were determined to monitor the influence of inulin on the composition of microbiota and their metabolic activity. Subjects consumed inulin or placebo with each of the main meals (total ca. 20 g/ d) for four weeks, separated by a two week washout period. Iron absorption was measured after three weeks of inulin and placebo consumption from a standard test meal of rice and vegetables. Mean fractional iron absorption from the test meal during the inulin period (15.2 %) was not significantly different from iron absorption during the placebo period (13.3 %). Inulin however significantly decreased fecal pH ($P< 0.001$) and increased bifidobacteria population ($P< 0.001$) as well as lactate ($P< 0.001$), but had no impact on fecal SCFA profile and total bacteria. Changes in lactate concentration and changes in acetate concentration were significantly correlated with changes in propionate ($P< 0.001$) and butyrate concentration ($P< 0.02$), respectively.

Thus, although inulin demonstrated a prebiotic activity, we were unable to show an increase in iron absorption measured by erythrocyte incorporation.

## Introduction

Iron deficiency (ID) is the most prevalent micronutrient deficiency worldwide, affecting nearly 2 billion people, mainly women and children in both developing and industrialized countries [1]. A major cause of ID is the regular consumption of diets based on plant foods and consequently low in bioavailable iron. [2]. Bioavailability of non-heme iron, providing most of the iron in a typical diet in developing countries, is strongly influenced by the concentration of enhancers and inhibitors [3]. Phytic acid is the most important iron absorption inhibitor, present at high levels in all major cereal grains and legume seeds [4-6]. Polyphenol compounds can be as potent as phytate in inhibiting iron absorption and do occur at high levels in beverages such as tea, coffee and red wine as well as in certain legumes, fruits and vegetables [7-10]. The main enhancer of non-heme iron absorption is ascorbic acid [11], which exerts its effect by reducing ferric iron to the ferrous form and by binding ferrous iron in a soluble chelate [12]. The only other widely accepted enhancer of iron absorption is muscle tissue, whose enhancing effect is commonly known as the “meat factor”. The effect has been demonstrated in several *in vivo* studies [13-15], but until today the identification of its structure and mechanism remains unclear [16].

Other potential enhancers of iron absorption are the non-digestible carbohydrates inulin and oligofructose, which are regarded as prebiotics since they selectively stimulate the growth and activity of specific bacteria beneficial for the human host [17]. Studies in rats [18] and pigs [19, 20] have reported enhanced iron absorption in combination with inulin and have proposed that the fermentation of inulin in the colon and the associated changes in the gut microbiota increase colonic iron absorption [21]. Although iron in humans is mainly absorbed in the duodenum, radioiron studies have demonstrated the ability of the human colon to absorb iron [22, 23]. Nevertheless previous human studies have failed to demonstrate an impact of inulin on iron absorption [24, 25]. However, a recent long term feeding study has reported 45 % less iron deficiency anemia in children fed an iron fortified milk enriched with oligosaccharides and *Bifidobacterium lactis* compared to children consuming the control fortified with iron only [26]. In the context of biofortification of staple foods [27], the concentration of inulin in wheat (1-4 %) [28] could be increased by the plant breeders and it has been suggested that inulin enriched wheat might improve iron nutrition in targeted populations and reduce the number of people with iron deficiency [19].

The following study used a stable iron isotope technique to investigate the influence of inulin on iron absorption from test meals consumed by women with low iron status. To monitor the effect of inulin on gut microbiota and metabolic activity, bifidobacteria population, total bacteria, fecal pH and fecal SCFA profile were measured in the faeces of subjects.

## Methods

### *Subjects*

One hundred-thirty-two women from the student and staff population of ETH Zurich and the University Hospital Zurich were screened for iron status (haemoglobin (Hb), serum ferritin (SF) and C-reactive protein (CRP)) as well as for body weight and height. Thirty-six apparently healthy, non-pregnant, non-lactating women with marginal iron stores (SF< 25 µg/L), aged between 18 and 40, and below 65 kg body weight were included in the study. Women with known metabolic chronic and gastrointestinal disease as well as woman on long-term medication were excluded. Intake of vitamin and mineral supplements as well as intake of pre- and probiotics was not allowed during the study period. No women were recruited who had donated blood or experienced significant blood loss within 6 months of the beginning of the study.

The experimental procedures were approved by the ethical committee of ETH Zurich and written informed consent was obtained from all study subjects before the investigation began.

### *Study design*

A randomized, double blind crossover design was used, in which each subject acted as her own control. Participants were randomly assigned to receive either inulin (Fibruline Instant; Cosucra Group Warcoing, Belgium; Inulin concentration in the product about 90 % with an average degree of polymerization of about 10) or an identical-appearing placebo (Maltodextrin; Blattmann Schweiz AG, Wädenswil, Schweiz) for 4 weeks followed by a 2-week washout period (Figure 1). After the washout period, each subject received the alternative treatment for 4 weeks. Subjects received a container of inulin or placebo for each treatment period. They were instructed to consume 3 measuring spoons (6-7 g) of inulin or placebo on a

daily basis. Inulin or placebo were dissolved in water and consumed with breakfast, lunch and dinner. Total inulin and placebo consumption was calculated by reweighing the container at the end of each period.

On day 22 of each treatment period (day 22; day 66), the subjects received two isotopically labeled test meals (Figure 1). Test meals were of moderate iron bioavailability and consisted of cooked rice (50g dry weight) and a pureed, boiled vegetable sauce (25g fresh weight; boiled for 4 h) containing Chinese cabbage, carrots, zucchini, onions, oil and salt. The labeled meals were administered in the morning between 0700 and 0900 after an overnight fast and a second meal three hours later. Subjects were not allowed to eat and drink between the test meals and three hours after the second meal. They consumed test meals including water (300 ml) completely in presence of the investigators. On the days of test meal administration, subjects consumed their inulin or placebo doses without food 1 hour before the test meals to avoid any interaction with iron from the test meal.

Blood was taken after an overnight fast for iron isotopic analysis 14 days after test meal administration (day 36, day 80; Figure 1). Iron absorption was calculated based on erythrocyte incorporation of iron stable isotope labels 14 d after intake of labeled test meals [29]. Faeces were collected on day 0, day 21, day 29, day 65 and day 72 (Figure 1) to measure faecal microbiota, pH and the short chain fatty acid profile.

Dietary assessment was done using a 3-day weighed food record in each of the two treatment periods. Dietary data obtained from the two records was entered into a nutrition software system (EBISpro for Windows 6.0, Dr. J. Erhardt, University of Hohenheim, Germany) for analysis.

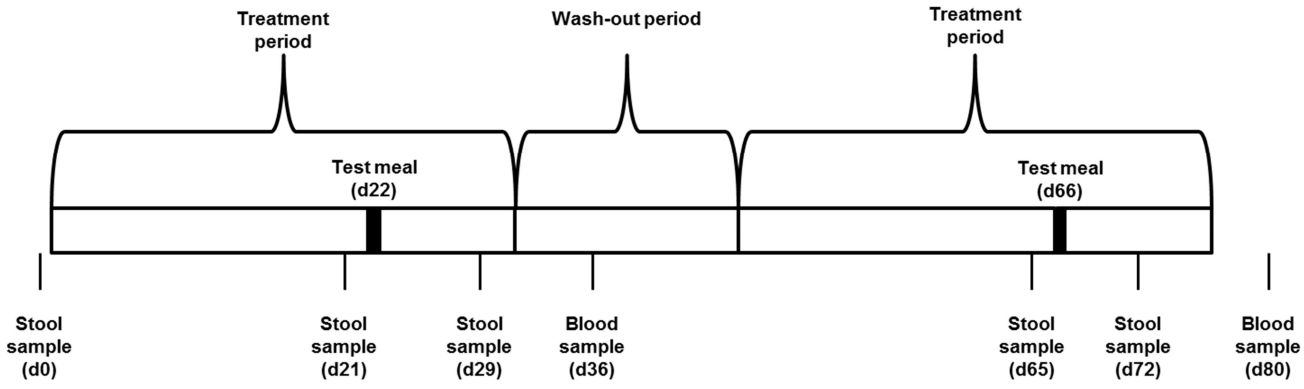


**Figure 1** Study design

***Stable isotope labels***

Isotopically-labeled $^{58}FeSO_4$ and $^{57}FeSO_4$ prepared from isotopically enriched elemental iron ($^{57}$Fe-metal: 97.8% enriched; $^{58}$Fe-metal: 99.5 % enriched; both Chemgas, France) by dissolution in 0.1 mol/L sulfuric acid. The solutions were flushed with argon to keep the Fe in the +II oxidation state. They were analyzed for iron isotopic composition and tracer iron concentration by reversed isotope dilution mass spectrometry using the experimental techniques outlined below.

***Analytical methods***

Whole blood samples were mineralized using an $HNO_3/H_2O_2$ mixture and microwave digestion followed by separation of the iron from the blood matrix by anion-exchange chromatography and a solvent/solvent extraction step into diethyl ether [30]. All isotopic analysis were performed by negative thermal ionization mass spectrometry (NTI-MS) using a magnetic sector field mass spectrometer (MAT 262; Finnigan MAT, Bremen, Germany) equipped with a multi-collector system for simultaneous ion beam detection [29, 30]. Venous blood samples were drawn in EDTA-treated tubes to determine iron status including Hb and SF. Blood samples were divided into aliquots for the analysis of Hb and isotopic composition and plasma was separated, aliquoted and frozen for the later analysis. Hb was measured with a Coulter Counter. SF and serum C-reactive protein (CRP) were measured on an IMMULITE® automatic system (DPC Bühlmann GmbH, Allschwil, CH).

Fecal samples for the analysis of fecal microbiota, pH and SCFA profile were freshly transferred by the subjects themselves to a tube containing a $CO_2$ generator system so as to create an anaerobic atmosphere (Microbiology Anaerocult A mini, Merk, Darmstadt, Germany). The faecal sample was stored in anaerobiosis at 4℃ for a maximum of 12 h until delivery to the laboratory. Immediately upon reception, faecal samples were aliquoted and either stored at -20℃ until the day of analysis or centrifuged at 14000 rpm for 120 min at 4℃. The obtained faecal water was carefully removed and stored at - 20℃ until analysis. The pH of faecal water was measured using a digital pH meter (Metrohm, Zofingen, Schweiz). HPLC (Hitachi LaChrome, Merck, Dietikon, Switzerland) measurements for SCFA (acetate, propionate, butyrate

and formate), iso-acids (iso-butyrate and iso-valerate) and lactate were performed as described previously [31].

***Nucleic acid extraction***

DNA was extracted from 100 mg feces using a FastDNA Spin Kit for Soil (Qbiogene AG, Basel, Switzerland). Quantification was carried out with a Nanodrop® ND- 1000 Spectrophotometer (Witec AG, Littau, Switzerland) at 260 nm.

***Quantitative PCR (qPCR) analysis***

DNA amplification and detection by qPCR was done with a 7500 Fast Real-Time PCR System (Applied Biosystems Europe BV, Zug, Switzerland) using optical-grade 96-well plates. Sample analysis was performed in a total volume of 25 µL using SYBR® Green PCR Master Mix (Applied Biosystems) containing 200 nM of the appropriate primers and 1 µl template DNA diluted 10- or 100- fold, depending on the targeted bacteria group. For the quantification of total bacteria, the primer Eub338F (5' ACT CCT ACG GGA GGC AGC AG 3') and Eub518R (5' ATT ACC GCG GCT GCT GG 3') were used. For the detection of *Bifidobacterium spp.* we used *the* primer xfp-fw (5' ATC TTC GGA CCB GAY GAG AC 3') and xfp-rv (5' CGA TVA CGT GVA CGA AGG AC 3').

Real-time qPCR conditions were kept at the presettings of the ABI PRISM 7500-PCR as described earlier [32].

***Calculation of Fe absorption***

The amounts of $^{57}$Fe and $^{58}$Fe isotopic labels in blood 14 d after administration of the test meals were calculated on the basis of the shift in iron isotope ratios and on the estimated amount of iron circulating in the body. Circulating iron was calculated based on the blood volume estimated from height and weight according to Brown et al. [33] and measured Hb concentration. The calculations were based on the principles of isotope dilution and took into account that iron isotopic labels were not monoisotopic [29]. For calculation of fractional absorption, 80% incorporation of the absorbed Fe into red blood cells was assumed.

### *Statistical analysis*

Analyses were conducted with SPSS statistical software (SPSS 19.0; SPSS Inc.) and Microsoft Office Excel 2003. Iron absorption values were log-transformed for statistical analysis and reconverted for reporting. Iron absorption values from the two different test treatments within the same participant were compared by a paired Student's t-test. Differences were considered significant at $P< 0.05$. The study was powered to resolve a 20 % difference in iron absorption between test meals using each volunteer as her own control.

For parameters that were not normally distributed (SCFA, pH, total bacteria, bifidobacteria, SF), multiple comparisons of values from the three experimental periods (baseline, inulin, placebo) within the same participants were done using Friedman followed by a Wilcoxon Signed-Rank Test with Bonferoni' s adjustment. Differences were considered significant at $P< 0.017$. A one-way ANOVA, followed by a post hoc Bonferroni's Test was used for comparisons of normally distributed values (CRP, Hb).

## Results

### *Subjects*

Two subjects dropped out of the study due to health issues and another 2 subjects were excluded from the evaluation due to the consumption of pre-and probiotics; 32 subjects completed the study.

At baseline, 2 out of the 32 study subjects had a hemoglobin concentration < 120 g/L. The median SF concentration was 14.2 µg/L (3.2- 24.5) and 17 subjects had a SF concentration < 15 µg/L with none above 25 µg/L. Eight women showed a slightly elevated CRP concentration of >3 mg/L at baseline but none had a CRP concentration >10 mg/L. The mean body mass index (BMI) was 21.5 ± 2.2 kg/m$^2$. Hb, SF and CRP concentrations and BMI did not change during the study.

### *Inulin and iron intake*

The average daily Fibruline Instant and placebo consumption during the treatment periods was 20.2 ± 2 g and 20.8 ± 3.2, respectively without any significant differences between subjects. Based on three day weighed records, inulin intake from meal components ranged between 0.4 and 4.6 g per day, mainly from wheat. Daily iron

consumption was estimated at 10.9 ± 3 mg. Both, inulin and iron intake did not differ between the inulin and placebo treatment periods.

### *Iron absorption*

Mean fractional iron absorption from the test meal given during the inulin period was 15.2 % (8.0; 28.9). The mean fractional iron absorption from the test meal given during the placebo period was 13.3 % (8.1; 24.3). Although the absorption was 14 % higher in the inulin period, this difference was not statistically significant (P= 0.11).

**Table 1** Fractional iron absorption of women who consumed inulin or placebo[1,2]

| n | Fractional iron absorption Inulin phase | Fractional iron absorption Placebo phase | Ratio[3] | p |
|---|---|---|---|---|
| | % | % | | |
| 33 | 15.2 (8.0; 28.9) | 13.3 (8.1; 24.3) | 1.14 | 0.11 |

[1]all meals contained either 2 mg $^{57}$Fe or 2 mg $^{58}$Fe
[2]Values are geometric means (range)
[3]Absorption ratio test meals administered in the inulin phase/ test meals administered in the placebo phase

***Gut microflora***

Total bacteria concentration at baseline was 11.2 (8.9- 12.3) log copies/ g feces and did not vary between stools collected at baseline and in the placebo and inulin periods. In contrast, stool bifidobacteria concentrations increased during the inulin administration from 9.6 (8.3- 10.6) to 10.5 (8.8-11.3) log copies /g feces (P< 0.001). Bifidobacteria concentration did not differ between baseline (9.6 log copies /g feces) and placebo (9.9 log copies /g feces; Figure 2).

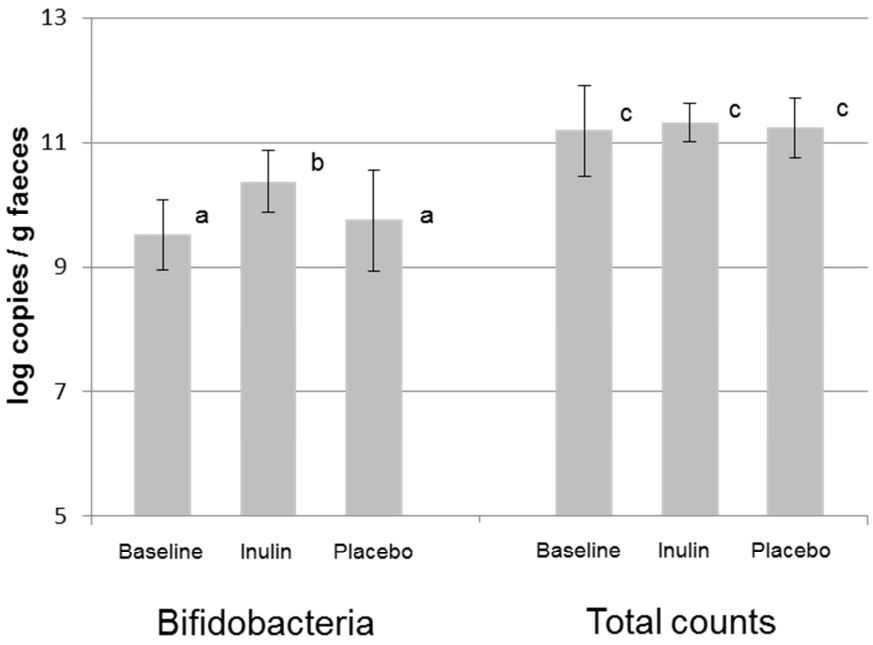


**Figure 2**: Log number of gene copies / g faeces of bifidobacteria and total bacteria (median) by real- time polymerase chain reaction performed with fecal DNA from faeces of subjects collected during baseline, placebo and inulin periods. Values without common letter differed significantly, P< 0.017 (Friedman Test, Wilcoxon Signed -Rank Test, Bonferoni Test).

Total SCFA concentration in fecal water, as well as the concentrations of acetate, butyrate, propionate and formate did not differ between stools collected in the baseline, inulin and placebo periods. Fecal concentration of iso-acids was also similar in stools at baseline and stools from the inulin and placebo periods. Lactate concentration was different in stools collected during the inulin period compared to stools collected during the placebo period (P< 0.005) or at baseline (P< 0.001). There was no significant difference in lactate in stool collected at baseline or during the placebo period (Table 2).

**Table 1** Total SCFA, SCFA (acetate, propionate, butyrate, formate), lactate, iso-butyrate and isovalerate concentration in mMol in human feces (n= 32)[1]

| | Total SCFA | Acetate | Propionate | Butyrate | Formate | Lactate | iso- butyrate | iso-valerate |
|---|---|---|---|---|---|---|---|---|
| Baseline | [a]120(39-207) | [a]65(15-113) | [a]20(12-90) | [a]19(5-66) | [a]0(0-18.7) | [a]0(0-1.9) | [a]3.0(0.9-8.3) | [a]3.0(0.9-8.3) |
| Inulin | [a]115(57-175) | [a]76(18-107) | [a]19(0-52) | [a]22(5-56) | [a]0(0-29.5) | [b]0(0-74) | [a]2(0-5.1) | [a]2.0(0-5.1) |
| Placebo | [a]112(35-230) | [a]72(16-121) | [a]21(10-72) | [a]21(9-48) | [a]0(0-17) | [a]0(0-5) | [a]3.3(1-7.7) | [a]3.3(1-7.7) |

[1] Median; range in parentheses. Values in a column with superscripts without common letter differ, *P*< 0.017 (Friedman's Test, Wilcoxon Test, Bonferroni)

Fecal pH during the inulin period was 6.5 (4.1- 8.7) which was significantly lower than fecal pH at baseline (P< 0.001) or during the placebo period (P< 0.005), which were 7.5 (5.5-8.6) and 7.6 (5.5-8.6) respectively. Baseline and placebo fecal pH values did not differ (Figure 3).

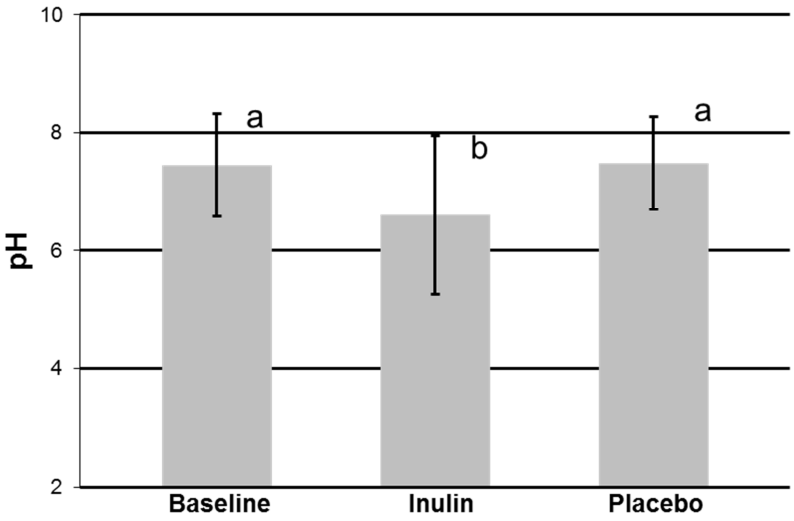


**Figure 3** Fecal pH of subjects from baseline, inulin and placebo period. Values without common letter differed significantly, P<0.017 (Friedman Test, Wilcoxon Signed -Rank Test, Bonferoni Test).

***Correlations between different study parameters***

In multivariate regressions the difference in bifidobacteria (P= 0.958), the difference in total bacteria (P= 0.168), the difference in lactate (P= 0.601), the difference in pH (P= 0.353) and the difference in total SCFA (P= 0.936) between inulin and placebo

period were no significant independent predictors of the difference in iron absorption between the inulin and placebo period.

There was a significant correlation between the difference in lactate concentration in the inulin and placebo period and the difference in propionate concentration (r= .743; P< 0.001) and a close to significant correlation between the difference in lactate concentration and the difference in acetate concentration (P= 0.086; r= .313) between the inulin and placebo period, but there was no correlation between the difference in lactate concentration and the difference in butyrate concentration(P= 0.795; r= .049). The difference in acetate concentration significantly correlated with the difference in butyrate concentration (P< 0.02; r= .427) and the difference in formiate concentration (P< 0.01; r= .463) between the inulin and placebo period.

**Discussion**

The consumption of inulin increased the numbers of bifidobacteria in the stool of study participants, indicating that inulin dose as well as study duration was sufficient for inulin to change the colonic environment in a way that could potentially increase colonic iron absorption. Nevertheless, we were not able to show a significant impact of inulin on erythrocyte incorporation of the stable iron isotopes administered with the test meals.

The daily consumption of about 20 g inulin for a period of 4 weeks caused a significant increase in the bifidobacteria population in the stool of the study participants [34-36] and probably led to a shift in the concentration of pH sensitive bacteria by the reduction of the pH from 7.5 to 6.5 [37]. The increase in bifidobacteria however was not unexpected since inulin has been shown to stimulate the growth and activity of bifidobacteria and protect humans from intestinal infections by suppressing the growth of potential pathogens and therefore is referred to as prebiotic [38]. The "bifidogenic effect" of inulin in humans has already been observed at much lower inulin concentrations than administered in the present study [34] and has been shown to be associated with an increased production of short chain fatty acids in rats [39] and *in vitro* [40]. It was not unexpected however that we failed to demonstrate an increase in stool SCFA in the inulin period of our study as more than 95 % of SCFA generated are absorbed rapidly in the colon and the final fecal output is generally low [41]. High concentrations of SCFA have been reported in human digesta soon after accidental death when the absorption processes have stopped but fermentation continues [42]. Cummings et al [42] reported that SCFA concentration increased from 1mmol/ kg digesta in the jejunum of deceased subjects to 13 mmol/ kg in the ileum, to 131 mmol/ kg in the caecum. SCFA concentrations fell slightly in the colon from 123 mmol/ kg in the ascending colon to 80 mmol/ kg in the descending colon, always correlating negatively with colonic pH, which demonstrates the ability of microorganism in the human colon to generate SCFA. The decrease in fecal pH, observed in our study did not correlate with fecal SCFA concentration but was most likely related to an increase in lactate and other metabolites (not measured) in the feces. For example inulin consumption has been shown to be associated with an increase in succinic acid and phosphate [39] and also changes in sodium bicarbonate concentration might have had an influence on colonic pH [43]. The 15% decrease in pH however indicates that the administered inulin reduced pH

throughout the whole colon, most likely strongest in the proximal part where higher concentrations of the substrate were present. A further indicator that inulin was degraded all along the colon is the high concentration of lactate found in the feces of the subjects. Lactate is an intermediate and not a fermentation end product [44] and its high concentration in the feces therefore suggests the degradation of inulin even in the distal colon of the participants.

However, it is well accepted that the major site for iron uptake in humans is the duodenum, an intestinal section where no inulin metabolization takes place. Early radioiron studies indicated that orally administered iron is absorbed in 2 phases. In these studies the larger iron fraction (60- 80 %) of total iron absorption was absorbed within the first 2h-4h, whereas the remaining iron was absorbed at a much slower rate over the next 22-48h [23, 45]. The site of the second phase of iron absorption is not totally clear yet and the authors speculated that it could be the slow release of iron stored temporarily in mucosal cells. However, a more likely explanation is colonic absorption [46], especially since about 90% of dietary iron is not absorbed in the duodenum and arrives in the colon. In 1963 Ohkarawa et al [22] first demonstrated, that humans can absorb iron from the colon. Fractional absorption of iron infused into the colon as ferrous chloride was 7% compared to 21 % fractional absorption from orally administered ferrous chloride, both measured as erythrocyte incorporation. For colonic absorption, as for duodenal absorption, iron bioavailability is highest in the ferrous form. However most stool iron is in the ferric form and ferric chloride infused into the colon has been shown to be absorbed only by 0.5% [22]. Further indicators for colonic iron absorption have been detected in the pig, rat and mouse, which have been shown to express the iron absorption proteins DMT1, ferroportin and hephaestin in their colon epithelial cells [47-50], although to a lower extent than the duodenum. Therefore it can be assumed that these proteins are also expressed in the mucosal cells of the human colon.

Animal studies indicate that low stool pH and low iron status favour colonic iron absorption [46] and for this reason we selected subjects of low iron status for the present study. Campos et al [51] reported that colonic iron absorption was up regulated in iron deficient rats and that this was due to an increase in both passive and active iron absorption. Passive absorption was assessed after the addition of 2,4 Dinitrophenol, an inhibitor of energy dependent absorption pathways. In the iron deficient rats, passive colonic absorption of calcium, zinc and copper was also

increased, and the authors suggested that this was due to an increased permeability of the colonic membrane due to iron deficiency.

Further evidence that iron deficiency upregulated colonic iron absorption, even to a greater extent than duodenal iron absorption came from the work of Chernelc et al [46] with dogs. After phlebotomizing the dogs in the study, colonic iron absorption increased from 2.5 % to 15 % compared to an increase in duodenal iron absorption from 8.6 % to 24 %. The same workers also reported a positive impact of low pH on colonic iron absorption. Iron absorption from an iron dose directly injected into the colon of dogs at pH 2 was almost double that at pH 6.

A decrease in colonic pH after the consumption of inulin has been reported in rats and is one of the proposed mechanisms by which inulin and oligofructose might enhance colonic iron absorption [18]. Linear regression analysis in our study showed no correlation between differences in iron absorption in the inulin and placebo period and differences in pH. However, Yasuda et al 2006 [19] found an increase in iron solubility in the stools of iron deficient pigs after feeding a diet containing 4% of a mixture of oligofructose and inulin, but with no changes in colonic pH. The same workers reported an inulin- induced decrease in the expression of inflammatory genes in a subsequent study with anemic piglets [52]. It is possible therefore that inulin consumption may reduce inflammatory cytokines and improve iron absorption by decreasing hepcidin transcription [53].

Tako et al reported increased expression of DMT1 and ferroportin in the duodenum of pigs after inulin supplementation and an increased DMT1, ferritin and TfR expression in the colon [20]. In a subsequent review the same group suggested the following ways by which inulin could increase colonic iron absorption: decreasing colonic pH, reducing ferric to ferrous state, stimulating the expression of genes coding for iron regulatory proteins and stimulating the proliferation of epithelial cells, thus providing a greater surface for iron absorption [21].

Despite these consistent demonstrations in rat and pig models showing that inulin increases colonic iron absorption, human studies have failed to confirm inulin as an enhancer of iron absorption. Coudray et al [25] investigated the effect of inulin on human iron absorption in a metabolic balance study in 9 healthy men receiving up to 40 g inulin per day. After an adaptation period of three weeks faeces and urine was collected for 8 days and blood samples were taken on day 25. The apparent iron absorption from the control meal was 21.8 % ± 12.3 % and did not further increase

with the inulin diet. One reason may be that iron bioavailability from the control diet without inulin was already very high in a group of iron replete men and therefore was unlike to be substantially further increased. A second study by van den Heuvel et al [24] used stable iron isotopes. In this study 15 g of inulin was consumed with a basal diet for a period of 21 days. They also reported no influence of inulin on erythrocyte incorporation of stable iron isotopes. However, the protocol was not ideal as ascorbic acid consumption was > 400 mg/ day and the study subjects were iron replete men with SF > 80 µg/L.

Our study is therefore the third human study to report no effect of inulin on iron absorption. Inulin consumption in our study did increase colonic bifidobacteria, which created an environment comparable to the long term study reporting a 45 % decrease in the prevalence of iron deficiency anemia in a group of children consuming *Bifidobacterium lactis* and oligofructose [26]. However, in contrast to our study a single bifidobacteria strain was administered indicating that specific bacteria strains might play a key role in iron metabolism.

While it seems probable that iron is absorbed in the colon in humans, the colonic absorption is likely to be a minor component of total iron absorption compared to duodenal absorption and thus difficult to quantify using the erythrocyte incorporation of isotopes. Our study included 32 subjects (instead of the normal 16) and was powered to detect a 20 % difference in iron absorption. Although iron absorption in the inulin period increased 14 % this difference was not statistically significant. While long term inulin consumption in combination with certain bifidobacteria strains might improve iron status [26] it seems unlikely that increased inulin levels in staple foods by biofortification could usefully improve iron absorption and iron status.

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# Conclusions and perspectives

Biofortification is a new and also very challenging approach to combat micronutrient deficiencies. To be successful many hurdles have to be overcome and many variables need to be controlled. Unlike to other interventions, biofortification faces people related as well as plant and environmental related challenges. The newly developed plant varieties have to exhibit a high concentration of bioavailable nutrients which must be stable over different environments, exhibit a high yield, resistance against pathogens and must be accepted by the consumers and producers (Bouis and Welch 2010, Nestel et al 2006). Environmental and plant related factors such as climate, soil and genetic variability might be the major bottlenecks to biofortification since they can be little influenced by human manipulation and have been shown to play a considerable role in terms of nutrient concentration. Genetic engineering should be considered as a possible approach for crops such as rice and wheat with insufficient genetic variability and/or high micronutrient losses during harvest and processing. However, biofortification is a rapidly developing field and is still in an early stage. There is still a long way to go until the first biofortified crop is disseminated and is demonstrated to successfully improve the nutritional status of a targeted population. Not until then will it be known whether biofortification can come up to expectations, also in terms of cost effectiveness and sustainability (Mayer et al 2008).

The thesis was designed to assist plant breeders in the development of their breeding strategies by focusing on compounds influencing iron bioavailability. Until now, the major focus of breeders has been on increasing the concentration of nutrients in the plant and less attention has been given their bioavailability. However, traditional plant breeding strategies which concentrate on increasing the levels of iron are fast reaching their limits due to limited genetic variability in the plant. Since iron absorption from plant based diets is generally low (Hurrell and Egli 2010), success of biofortification might become more likely if bioavailability and concentration of iron are increased simultaneously.

Beans are a major staple food for more than 300 million people and therefore are a targeted crop of biofortification initiatives. It is planned to release the first biofortified

varieties in D.R. Congo and Rwanda in 2012 (HarvestPlus 2009). Beans are in terms of iron concentration, genetic variability and micronutrient retention a promising crop for iron biofortification. Newly developed varieties exhibit iron concentrations of more than 10 mg/ 100g, which is a level considered to have the potential to significantly increase the iron status of a bean consuming population (Blair et al 2010). The preliminary iron target level was set to 9.4 mg/100 g, assuming an iron bioavailability of 5 % (Bouis and Welch 2010). However, reported iron bioavailability is with 2%-3% far lower (Beiseigel et al 2007, Donangelo et al 2003, Lynch et al 1984), most likely due to the high concentration of polyphenols and phytic acid. It has been shown that the bean polyphenol concentration usually differs strongly, depending on the bean color and variety, but phytic acid is constantly high (Anton et al 2008, Towo et al 2003) and positively correlates with iron concentration (Hoppler et al unpublished). The inhibiting effect of polyphenols on iron absorption has been demonstrated, but the capability of complex formation with iron in the intestine and thereby the reduction of iron uptake into the body depends on their structure (Hurrell et al 1999, Tuntipopipat et al 2006). This project was the first to confirm the negative effect of bean polyphenols on iron absorption. Moreover our results revealed that reducing either polyphenols or phytic acid alone will only have moderate effects or no effects on iron bioavailability. The negative impact of the two inhibitors on iron absorption has been shown not to be additive, most likely because they were both present in the bean in molar excess compared to iron. However, it has to be noted that the iron absorption from the beans we administered in Rwanda ranged from about 3.5 % to 7.4 %, which is very encouraging for biofortification.

Testing the usefulness of a biofortified high iron bean in a bean consuming population surprisingly revealed that the additional iron bred into the bean did not increase the amount of iron absorbed by the subjects. The amount of iron absorbed from the biofortified bean and the reference bean was, with about 200 µg/ meal, similar, although the biofortified bean contained almost double the amount of iron. Polyphenol concentration as well as the phytic acid to iron molar ratio, which have been shown to be determinants of relative iron bioavailability were comparable in both beans. We expected slightly decreased relative iron absorption from the biofortified high iron bean, but still a significant dose dependent increase in total iron absorbed since subjects were iron deficient (Cook et al 1973, Hallberg et al 1998).

We consider it most likely that the presence of high levels of phytate, polyphenols and inhibitory proteins in the high iron bean reduced the amount of bioavailable iron. Iron absorption from the high iron bean in our study was only 3.5 %, almost 30 % below the requested minimum amount calculated to improve the nutritional status of a targeted population. To increase iron absorption from high iron beans we suggest to select for high iron beans with low polyphenol and low phytic acid concentration. However, it must be noted that our studies were short term multiple meal isotope studies and that it still needs to be clarified if polyphenols and phytic acid maintain their strong negative impact on iron absorption over the long term or if, in the real life situation, people can upregulate iron absorption over time to meet their iron requirements.

An exciting new possible solution to alleviate the problems associated with phytic acid might be a recently isolated low phytic acid (lpa) mutant. This mutant shows a 90 % reduction in phytic acid. The mutant has normal phosphate levels and exhibits, in contrary to other lpa mutants, a high yield and germination rate (Campion et al 2009).

Other workers detected that the low polyphenol concentrations found in white beans are not related to a lack of pigments. Furthermore they reported that the variation in polyphenol levels within a single colour class could be higher than between the different colour classes. This would make a selection for low polyphenol traits in the different bean colour classes possible. Crossing the low phytic acid mutant with bean varieties low in polyphenols and high in iron might lead to the development of a variety with the favored characteristics. However, the planned release of a biofortified bean variety in 2012 with a high concentration of bioavailable iron which is stable over different environments and seasons seems to be optimistic and it may not be feasible by that time to confirm that the extra iron will increase the amount of iron absorbed and improve iron status.

Inulin, which is present in considerable amounts in wheat (1-4 %) (Nair et al 2010) and might be further increased by biofortification, was tested for its potential as iron absorption enhancer. We confirmed the ability of inulin to act as a prebiotic by showing a significant increase in bifidobacteria population, which are known to be beneficial for the human host (Roberfroid 2006). But although inulin and oligofructose

have been reported to exhibit a positive effect on iron absorption in animals (Ohta et al 1995, Tako et al 2008, Yasuda et al 2006) we were not able to show a similar effect in humans. One problem of our study was that the methodology used was only powered to detect a 20 % difference in iron absorption and the increased we found was 14 % which was not significant. If inulin had any effect on iron absorption, it remained undetected and would not be expected to be a major iron absorption enhancer. In terms of biofortification, it also has to be taken into consideration that the amounts of inulin (ca. 20g/d) consumed in the study will rarely if ever be reached with the consumption of inulin enriched wheat or any other stable crop. We cannot therefore recommend inulin as a possible compound for iron biofortification. It might be more suitable as additive to sprinkles in in-home fortification programs. The stimulation of bifidobacteria by the consumption of inulin has been shown to suppress the growth of potential pathogens (Roberfroid 2006). Administering inulin within in-home fortification for example might reduce the prevalence of pathogens and with it the prevalence of intestinal infections, mucosal inflammation and diarrhea (Welters et al 2002). This would be beneficial for iron absorption since inflammation is known to decrease via an increase in hepcidin synthesis (Hentze et al 2010).

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# Curriculum vitae

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