Barley- and Legume Products Beneficially Affect Metabolic Responses and Appetite Regulation

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Barley- and legume products beneficially affect metabolic responses and appetite regulation

Implicating a role of gut fermentation in an over-night perspective

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Contents

Abstract

The role of dietary fibre (DF) in disease prevention has been extensively investigated and prospective studies observed that increased DF intake decreased the risk of cardiovascular disease (CVD), reduced the risk of weight gain as well as the risk of type 2 diabetes (T2D). Also a diet characterized by low glycaemic index (GI) has been shown to reduce the risk of T2D and CVD. More recently, evidence have accumulated suggesting that obesity, T2D and CVD may stem from a high-fat diet induced metabolic endotoxemia, implicating a role of the composition of gut microbiota. Mechanisms behind observed health merits of DF-rich foods suggests e.g. delayed digestion and absorption or increased gut fermentation of indigestible carbohydrates.

The present thesis studies the potential impact of indigestible carbohydrates intrinsic to barley kernel- and legume products on cardiometabolic risk factors and appetite regulation using a semi-acute meal study approach in healthy young or elderly subjects. The work described is based on randomized cross-over studies, and metabolic responses were measured in an over-night perspective, i.e. from evening meal to breakfast. The possible link between gut fermentation of indigestible carbohydrates and metabolic responses was evaluated from analysis of breath hydrogen (H_2) and circulating levels of short-chain fatty acids (SCFA).

Healthy young subjects were provided a late evening meal with boiled barley kernels (BK) or reference white wheat brad (WWB). At a subsequent breakfast, decreased blood (b-) glucose responses and increased concentrations of plasma (p-) glucagon-like peptide (GLP)-1 were observed after the BK evening meal, compared with WWB. The decreased b-glucose- and increased p-GLP-1 responses were maintained during the experimental day, 10.5-16 hours (h) after intake of the BK evening meal. In addition, decreased voluntary energy intake at a lunch meal consumed approximately 14 h after BK was registered while at the same time promoting decreased subjective ratings of hunger over the entire experimental day (10.5-16 h), compared to after WWB evening meal. Further, higher levels of gut fermentation metabolites, i.e. serum (s-) SCFA, and breath H_2, were

observed at breakfast after BK evening meal, compared to WWB. Supportive of a link between gut microbial metabolism and appetite regulation, p-GLP-1 levels were positively correlated to s-propionate, and inversely related to subjective feelings of hunger and desire to eat, respectively.

The impact of barley kernel based bread (BB) on glycaemia and appetite regulation were evaluated in elderly subjects (50-70 years) after three days intake of BB, as opposed to three days intake of WWB. At a subsequent standardized breakfast on day four, decreased b-glucose- and s-insulin responses were observed, and a measure of insulin sensitivity ($ISI_{composite}$) was improved. The metabolic benefits of BB in an over-night perspective previously observed in young adults (20-35 years) were thus also present among a more mature study cohort (50-70 years). The results are among the first to show that a meal rich in intrinsic indigestible carbohydrates promotes parallel increase in plasma levels of gut-derived hormones p-GLP-1, p-peptide YY (PYY) and p-GLP-2 in healthy humans. This is of potential importance in relation to facilitated glucose- and appetite regulation, as well as reduced sub-clinical inflammation. The increase in p-GLP-2 might be considered beneficial considering its role in maintaining integrity of the intestinal epithelium, in favor of a lowered inflammatory tonus. In addition, three days of BB compared to WWB stimulated gut microbial metabolism as indicated by increased levels of s-SCFA and breath H_2. The potential interplay between gut fermentation and host metabolism was supported by a positive correlation between total s-SCFA; and p-GLP-1 or p-PYY, respectively.

It was hypothesized that intake of commercially available probiotics could interfere with gut microbiota derived mechanisms for observed benefits of barley kernel products on metabolism and appetite regulation. Healthy young subjects were provided with BB or WWB during a four day intervention. BB intervention was performed twice, once including a dietary background of a mixture of probiotics (BB(+)) and once with placebo (BB(-)). The WWB intervention was performed with placebo background (WWB(-)). At a subsequent standardized breakfast, BB interventions increased p-GLP-1 levels and increased breath H_2 both with and without probiotic supplementation, compared to WWB(-). Decreased glycaemic responses were observed after BB(-) but not after BB(+), compared to WWB(-). However, not only p-GLP-2 but also p-glucagon and s-PAI-1 were increased after BB(+) compared to BB(-). It is concluded that with the exception of increased GLP-2 concentrations, overall outcome of the BB intervention was not

enhanced by a dietary background of common probiotics; rather, the benefits on glycaemic regulation were to some extent blunted.

Legumes, in conformity with barley kernel products, are naturally rich in DF, including RS. However the DF components differ. Thus, legumes are rich in raffinose-oligosaccharides and galactomannans, whereas barley is especially acknowledged for its high content of β-glucans. It was hypothesized that the DF present in legumes could promote gut fermentation and mediate benefits on glucose- and appetite regulation, as previously observed for barley kernel products. Boiled beans of different varieties (red, white, brown and black) or chickpeas were given to healthy young subjects as a late evening meal. WWB was included as a reference evening meal. A late-evening meal of boiled brown beans (BrB) beneficially improved glucose tolerance at a subsequent standardized breakfast, as judged from lowered b-glucose- and s-insulin responses, as compared to WWB evening meal. Having chickpeas in the evening significantly increased perceived feeling of satiety at fasting in the morning compared to WWB evening meal. All legumes increased gut fermentative activity 11-14 h after intake as indicated by increased breath H_2. Further, investigations with respect to cardiometabolic risk markers were performed after BrB only. The evening meal with BrB increased insulin sensitivity index ($ISI_{composite}$), increased s-SCFA and promoted higher levels of satiety hormones (p-PYY and p-oxyntomodulin (OXM)), reduced the orexigenic peptide ghrelin in plasma and decreased markers of inflammation (s-IL-6 and s-IL-18) in response to the subsequent breakfast, compared with WWB evening meal. Additionally, the BrB evening meal stimulated higher p-GLP-2 in the late postprandial phase at the standardized breakfast. Inverse relations between willingness to eat and p-PYY and p-GLP-2, respectively, were found and supporting a beneficial role of BrB in appetite regulation.

In summary, barley kernel- and legume products rich in DF, were found to induce metabolic advantages, as indicated by decreased glycaemia, increased insulin sensitivity, beneficial effects on appetite regulatory hormones and decreased voluntary food intake at subsequent meals. The results are supportive of a mechanism whereby indigestible carbohydrates may be involved in positive health outcomes, with respect to obesity, T2D and CVD, through mechanisms involving fermentation. The results provide interesting possibilities to develop new foods containing specific indigestible carbohydrate substrates capable of addressing the gut microbiota – host metabolism cross-talk.

Populärvetenskaplig sammanfattning

Resultaten i denna avhandling visar att kvällsmåltider baserade på hela kärnor av korn (kokta kornkärnor, kornkärnbröd), har positiva effekter på blodsocker- och aptitregleringen vid den följande frukostmåltiden, jämfört med en kvällsmåltid av vitt bröd. Även ett sent kvällsmål med bruna bönor hade liknande effekter. Vidare visade resultaten att en kvällsmåltid med bruna bönor kan sänka inflammationsgraden under efterföljande förmiddag. Resultaten är viktiga då de visar att intag av vissa kostfiberrika livsmedel med lågt glykemiskt index (GI) gynnsamt kan påverka riskfaktorer för uppkomst av diabetes typ 2 och hjärtkärlsjukdomar. Den gynnsamma effekten av kornkärnbaserade produkter och baljväxter, t.ex. bruna bönor, föreslås i denna avhandling vara kopplade till kolonfermentering av odigererbara substrat, dvs. kostfibrer. Samtliga studier i avhandlingen utfördes på friska försökspersoner i ett övernattenperspektiv, vilket betyder att en testmåltid intogs på kvällen och testning utfördes vid en standardiserad frukost som serverades ca 10 timmar därefter.

Bakgrunden till denna avhandling är den kraftiga ökningen av fetma och relaterade sjukdomar såsom diabetes typ 2 och hjärtkärlsjukdom, både i Sverige och globalt. Utvecklingen ser ut att fortsätta och drabbar dessutom alltmer även barn och tonåringar. Förebyggande arbete för att motverka denna utveckling är därför av stor vikt. Världshälsoorganisationen (WHO) har föreslagit att en hälsosammare kost är en av de viktigaste åtgärderna. Befolkningsstudier visar att det finns ett samband mellan ett ökat inslag av kostfiber respektive låg-GI livsmedel i kosten och en minskad förekomst av fetma och diabetes typ 2. Dessa studier talar också för en skyddande effekt av en kost rik på baljväxter. Syftet mad avhandlingen är att studera kostfiberrika låg-GI livsmedel baserade på hela kärnor av korn respektive baljväxter utifrån deras förmåga att dämpa olika riskmarkörer kopplade till fetma, diabetes typ 2 och relaterade sjukdomar.

Återkommande och långvarigt höga blodsockersvar stimulerar en låggradig inflammation vilket bidrar till en ökad risk för hjärtkärlsjukdom. Låggradig inflammation är en gemensam nämnare vid t.ex. fetma och diabetes typ 2. Inflammationsprocessen kan även

stimuleras via en obalans i tarmfloran, d.v.s. sammansättningen på de bakterier som lever i tarmen. Resultaten i avhandlingen visar att hela kokta kornkärnor, eller bruna bönor i ett kvällsmål, minskade blodsockerstegringen vid den efterföljande frukosten, och ökade halten av tarmhormon som är involverade i blodsocker- och aptitreglering (t ex GLP-1, PYY och GLP-2) hos unga friska försökspersoner. Effekten kunde sitta i upp till ca 16 timmar efter kvällsmåltiden. Dessutom sågs en sänkning av inflammationsmarkörer (IL-6 och IL-18) efter bruna bönor. En liknande effekt på riskmarkörer sågs även hos friska medelålders personer (50-70 år).

De gynnsamma effekterna på ämnesomsättningen i ett ”över-natten”-perspektiv föreslås härröra från aktivering av tarmfloran av de kostfibrer som finns i testprodukterna. Denna aktivering bildar gynnsamma metaboliter i tarmen, t ex kortkedjiga fettsyror, som i denna avhandling föreslås påverka frisättning av tarmhormon, med positiva effekter på blodsockret- och aptitregleringen, samt föreslås även kunna stärka tarmens barriär och därmed minska inflammationsdrivande komponenter i blodet. Dessutom, försökspersonerna kände sig mindre hungriga under dagen när de ätit kornkärnsprodukter eller bruna bönor som kvällsmål, dessutom åt de mindre till lunch när de ätit kokta kornkärnor kvällen innan.

I en uppföljande studie studerades om de positiva effekterna på ämnesomsättningen och aptitregleringen som visats efter kornkärnbaserade produkter och bruna bönor påverkas av samtidigt intag av kommersiellt tillgängliga probiotiska bakterier. Resultaten visar att tillskott av probiotika inte gav någon ytterligare effekt till den tidigare observerade effekten på glukostoleransen och GLP-1 i blodet. GLP-1 anses vara ett viktigt ”anti-diabetiskt” hormon. Däremot blev resultaten på andra riskmarkörer något skiftande. Koncentrationerna av GLP-2 samt PAI-1, en markör för hjärtkärlsjukdom, ökade. Detta gör att det i nuläget är svårt att dra några slutsatser angående effekter av en kombination kornkärnsprodukter och de probiotiska bakterier som testades.

Resultaten visar att vissa fiberrika livsmedel, t ex kokta kornkärnor och bruna bönor, kan förbättra blodsockerreglering och insulinkänslighet, dämpa inflammation och underlätta aptitreglering i en tidsperiod på upp till 16 timmar. Både intakt struktur, sort och mängd kostfiber är avgörande. Resultaten visar på en potential för odigererbara kolhydrater vid utveckling av livsmedel med antidiabetiska och viktreglerande egenskaper.

List of papers

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals. The papers are appended at the end of the thesis.

Paper I **Effects of indigestible carbohydrates in barley on glucose metabolism, appetite and voluntary food intake over 16 h in healthy adults**

E. V. Johansson, A. C. Nilsson, E. M. Östman, I. M. E. Björck
Nutrition Journal 2013 12:46

Paper II **Increased concentrations of gut hormones and insulin sensitivity index following three days intervention with a cereal product rich in indigestible carbohydrates in healthy middle aged subjects; a randomized cross-over study**

E. V. Johansson, I. M. E. Björck, A. C. Nilsson
Submitted Manuscript

Paper III **Effects of a Brown Beans Evening Meal on Metabolic Risk Markers and Appetite Regulating Hormones at a Subsequent Standardized Breakfast: A Randomized Cross-Over Study**

A. Nilsson, E. Johansson, L. Ekström, I. Björck
PLoS ONE (2013) 8(4): e59985

Paper IV **Over-night metabolic effects of barley kernel products with or without supplementation with some commercially available probiotics**

E. V. Johansson, I. M. E. Björck, A. C. Nilsson
Manuscript

The work presented within this thesis was financed by the Antidiabetic Food Centre, a VINNOVA VINN Excellence Centre at Lund University

The author´s contributions

Paper I

The author, E. Johansson, coordinated the study, performed the experimental work, was responsible for the analysis and evaluation of the results and for writing the manuscript.

Paper II

The author, E. Johansson, was involved in the study design, coordinated the study, performed the experimental work, and was responsible for the analysis and evaluation of the results and for writing the manuscript.

Paper III

The author, E. Johansson, took part in the study design, coordinated the study, performed the experimental work, and was involved in statistical analysis of the data and in writing of the manuscript.

Paper IV

The author, E. Johansson, was involved in the study design, coordinated the study, performed the experimental work, analyzed and evaluated the results and was responsible for writing the manuscript.

Abbreviations

BB – barley kernel based bread

BCFA – branched chain fatty acids

BK – boiled barley kernels

BMI – body mass index

BrB – boiled brown beans

b – whole blood

CNS – central nervous system

CVD – cardiovascular disease

CRP – C-reactive protein

DF – dietary fibre

DP – degree of polymerization

dm – dry matter

FFA – free fatty acids

GI – glycaemic index

GLM – general linear model

GLP – glucagon-like peptide

GP2 – glycaemic profile

HOMA-IR – homeostatic model assessment of insulin resistance

H_2 – hydrogen

h – hours

(i)AUC – (incremental) Area under the curve

iPeak – maximum postprandial increase from baseline

IL – interleukin

$ISI_{composite}$ – insulin sensitivity index

LPS – lipopolysasaccharides

MetS – Metabolic Syndrome

NSP – non-starch polysaccharides

OXM – oxyntomodulin

PAI – plasminogen activator inhibitor

p – plasma

PYY – peptide YY

RS – resistant starch

SCFA – short chain fatty acids

s – serum

T2D – Type 2 Diabetes

TNF-α – Tumor necrosis factor alpha

VAS – visual analogue scale

WWB – white wheat bread

WHO – World Health Organization

Introduction

Obesity is considered a major risk factor for several conditions of public health concern, including diseases such as cardiovascular diseases (CVD) and type 2 diabetes (T2D)[1, 2]. Generally, obesity results from an excess intake of calories. However, more recently qualitative aspects of foods, in addition e.g. the relative contribution of energy from protein, fat and carbohydrates are also being discussed, and there is emerging knowledge that the gut microbiota may affect the endocannabinoid system tonus with potential influence on adipogenesis[3]. Physiological control systems are present to maintain homeostasis between energy intake and energy expenditure, and dysfunctions in this system are contributing factors in the development of obesity. Obesity is defined as having a body mass index (BMI, kg/m^2) $\geq$ 30 and overweight and obesity are together characterized by the World Health Organization (WHO) as abnormal or excessive fat accumulation that may impair health[1]. Strategies to reduce overweight and obesity have been advocated by the WHO and includes e.g. limitations in energy intake from total fat and sugar, and increased consumption of e.g. legumes, whole grain (WG) and vegetables[1]. However, the prevalence of obesity is rapidly increasing, and about 65% of the world's population lives in countries where overweight and obesity kills more people than underweight. In 2008 the prevalence of obese subjects in the world reached above 10%[1]. Alarmingly, the prevalence of overweight among younger subjects is also increasing worldwide and WHO states that more than 40 million children under the age of five were overweight in 2011[1]. In Sweden, approximately 55% of men and 40% of women among the adult population are either overweight or obese[4] and 17% of schoolchildren between the age of 7-9 years, was reported to be overweight or obese in 2008[5]

The prevention of metabolic disorders such as obesity, T2D and CVD is highly needed. One such strategy involve the maintenance of optimal glycaemic control. The importance of glycaemic control has recently been emphasized and a diet characterized by low glycaemic index (GI)/glycaemic load reduces the risk of T2D[6, 7]. In all diabetes patients, therapy is focusing on maintaining glycaemic levels as close to the non-diabetic range as

possible[8]. A decreased glycaemia, involving lowered fasting glucose levels as well as lowered post-meal oscillations, is considered important to decrease risk of diabetic complications[9, 10]. Oxidative stress induced by recurrent postprandial hyperglycaemia plays a key role in the onset of insulin resistance, possibly through mechanisms involving impairment of insulin signaling in insulin-sensitive tissues by pro-inflammatory cytokines[11]. Interestingly, not only in diabetics[12], but also in young healthy subjects, more elevated post-prandial glycemic excursions may promote an increased inflammatory state[13, 14]. Thus strategies targeting glycaemia appears to be a useful approach not only in diabetics but also in order to prevent the onset of metabolic disorders, in apparently healthy subjects[9]

The global prevalence of T2D and obesity is predicted to continue to rise manifold. However, a reassuring thought in this discouraging context is that metabolic disorders to a large extent are preventable. It is therefore of paramount importance to investigate different possibilities to prevent the development of metabolic disorders linked to the MetS, and in this context, life-style modifications like physical- and in particular dietary interventions are suggested to be of great importance[1]. Preventive strategies, should therefore preferably include dietary measures, and are urgently needed from a public health perspective. Of relevance in this context, is that epidemiological studies which indicate that a diet rich in dietary fibre (DF) and low GI foods is inversely related to the development of T2D[15, 16]. Furthermore, the prevalence of MetS, inflammation and obesity has been reported to decrease with increased DF intake[17]. WG have shown protective properties against T2D[18, 19] and CVD[20], and are associated with a lower body weight (i.e. BMI)[21-23]. Both legumes and kernel based WG cereals represent low-GI, DF-rich foods and it could be suggested that an increased intake of such foods may result in cardiometabolic benefits.

Background

Obesity and the metabolic syndrome

The metabolic syndrome (MetS), or the insulin resistance syndrome, has been defined and named differently over time[24-27]. Obesity is central, but the syndrome represents a cluster of interconnected factors that increase the risk of CVD and T2D[28]. The MetS and related disorders are increasing in prevalence, and constitute major health challenges worldwide. In agreement with the epidemic features of the obesity burden, the prevalence of T2D is predicted to increase worldwide from 366 million in 2011 to 552 million by year 2030[29] and WHO states that the deaths due to diabetes will increase by two thirds between 2008 and 2030[30]. In obese young subjects in the U.S, associations were reported between increased prevalence of the MetS and increased obesity and insulin resistance[31]. The development of MetS is a result of a complex interaction between genetic, metabolic, and environmental factors, such as dietary- and physical habits[32] and the prevalence of the syndrome varies in different populations. For example, the criteria for the MetS was fulfilled in more than 40% of the adult population in certain areas in Puerto Rico[33], >30% among residents of Nairobi, Kenya[34] and approximately 22% in the adult U.S. population[35]. In Sweden, the prevalence of MetS among 50-year-olds varied between 11-16% in women and between 16-26% in men depending on which definition was used[36].

In a strict sense, the MetS is defined by the presence of any 3 out of 5 risk factors related to waist circumferences, blood pressure, triacylglycerides, fasting blood glucose and reduced HDL-cholesterol (Table 1)[28]. The diagnosis criteria are intended for use in clinical practice, rather than for research purposes, and as evident from the table, measures of e.g. insulin resistance are not included. Despite this, subjects with insulin resistance have up to fivefold higher risk to develop T2D than healthy individuals[26] and insulin resistance is acknowledged as an important causative factor for MetS-related components[37]. Thus, measurements of glucose tolerance and insulin sensitivity are important additional criteria for research purposes[37]. Also other markers with emerging

evidence for risk prediction of MetS-related diseases, such as e.g. markers of sub-clinical inflammation, are lacking in the current definitions. Thus, sub-clinical inflammation is increasingly acknowledged as a feature of relevance for the etiology of MetS-linked disorders such as T2D and CVD[38, 39]. Accordingly, when comparing the Framingham and Reynolds risk scores, respectively, for global cardiovascular risk prediction, Reynolds risk score – which includes also the inflammatory marker CRP in the algorithm – was more discriminating[40].

Table 1. Criteria for the MetS, with any 3 of 5 risk factors constituting the diagnosis[1].

Risk factors	Categorical Cut Points
Elevated waist circumference*	Men ≥ 94 cm; Women ≥ 80 cm
Elevated triglycerides	≥ 150 mg/dL (1.7 mmol/L)
Reduced HDL-cholesterol	Men > 40 mg/dL (1.0 mmol/L); Women > 50 mg/dL (1.3 mmol/L)
Elevated blood pressure	Systolic ≥ 130 and/or diastolic ≥ 85 mm Hg
Elevated fasting glucose	≥ 100 mg/dL (5.6 mmol/L)

* According to IDF[41]
[1]Modulated from Alberti *et. al.* (2009)[28]

Sub-clinical inflammation

Sub-clinical or low-grade inflammation is a condition triggered by e.g. nutrient over-loads and it is closely associated with obesity, insulin resistance and T2D[42]. Observational studies indicate that intake of WG or DF appears to be inversely associated with markers of low-grade inflammation, such as e.g. C-reactive protein (CRP)[17, 43, 44]. In general, dietary patterns rich in "healthy" components like fruit, vegetables, legumes, fish, poultry and WG indicates benefits in relation to markers of systemic inflammation[45]. On the contrary, diets which are high in e.g. refined carbohydrates, sugar and saturated fatty acids, and are low in DF from fruits, vegetables and WG, may act to induce an inflammatory response[46]. Further, longer term intervention with low-GI diets showed benefits on inflammatory markers in a meta-analysis of randomized controlled trials[47].

The role of inflammation in e.g. obesity-linked insulin resistance and hyperglycaemia has even been hypothesized as a primary cause rather than a consequence[48]. Hence, chronic, low-grade inflammation is considered a risk factor for the MetS[49]. Adipose tissue is considered as the largest endocrine gland in the body and secretes various adipokines such as hormones involved in e.g. insulin sensitivity (leptin), acute-phase proteins (e.g. plasminogen activator inhibitor (PAI)-1), and cytokines such as interleukin (IL)-6 and IL-18, into the systemic circulation. It has been observed that elevated pro-inflammatory mediators, such as tumor necrosis factor (TNF)-α, IL-6 and CRP are associated with e.g. increased BMI[49].

A pro-inflammatory state may also emanate from an imbalance of the gut eco-system mediated by e.g. dietary modifications, so called metabolic endotoxemia (see *Metabolic Endotoxemia*, page 24). It can thus be suggested that dietary components play a role in systemic inflammation, with potential impact on MetS-associated disorders.

Glycaemic regulation

The glycaemic index originally introduced by Jenkins *et al.* (1981) describes the postprandial glucose response of carbohydrate-rich foods[50]. The GI represents the incremental area under the glycaemic-response curve (iAUC) for a test food expressed as percentage of the response to a carbohydrate equivalent amount of a standard food, e.g. oral glucose or white wheat bread (WWB), measured during 2 hours (h) after consumption[51]. In a meta-analysis of 37 prospective observational studies it was observed that low-GI diets decreased the risk of T2D and CVD[52]. Further, analysis of long-term effects of low-GI diets in obese or overweight subjects suggests that low-GI diets constitutes a valuable tool in primary prevention of obesity[47]. GI has thus been put forward as helpful in order to classify foods according to their health-promoting value with respect MetS-related diseases.

The mechanisms whereby carbohydrate-rich food influence acute glycaemia relates to the rate of digestion and absorption and involves both physiological factors, such as e.g. enzymatic availability, gastric emptying rate, and food properties such as food structure, e.g. botanical, physical and/or chemical structure[16, 53, 54]. Legumes, pasta and boiled barley kernels are examples of low-GI (< 55) foods[55]. Foods with lowered glycaemia in the acute

time perspective after intake, also exert favorable effects on glycaemia at the next meal in healthy subjects, e.g. from breakfast to lunch, or from evening dinner to subsequent breakfast, the so-called second-meal effect[56-59]. Several mechanisms are discussed in relation to improved second-meal glucose tolerance of low-GI foods. For instance, it has been suggested that improved glucose response in the time perspective from breakfast to lunch (4 h) emanates from improved insulin action, possible due to a prolonged starch digestion[58] and suppression of free fatty acids (FFA) after intake of low-GI foods[57, 60, 61]. However, other contributing factors, such as the presence of fermentative carbohydrates, are probably involved in the second-meal effect reported in an extended time perspective, i.e. from evening meal to subsequent breakfast (10 h)[59]. Granfeldt *et al.* (2006) showed that improved glycaemia was observed in healthy subjects at a standardized breakfast after a barley kernel evening meal but not after a white spaghetti evening meal, even though both evening meals were of similar and low GI[62]. The differences in metabolic responses in the over-night perspective were suggested to relate to the differences in content of indigestible carbohydrates in the evening meal. The barley kernel (BK) evening meal contained importantly higher amounts of indigestible and fermentable carbohydrates such as non-starch polysaccharides (NSP) and resistant starch (RS) compared to the spaghetti evening meal. On the contrary, flour-based white wheat bread with added barley NSP or high amylose maize RS corresponding to the NSP or RS content in BK did not improve the metabolic response in an over-night perspective[63, 64]. Nor did a 100% WG barley flour porridge induce benefits on glucose tolerance at a standardized breakfast[63]. In contrast, the simultaneous supplementation to white wheat bread with barley NSP and high maize RS improved glycaemic regulation to a similar extent as did the BK evening meal[64]. These results are indicative of that a specific combination of DF fractions, i.e. both NSP and RS is mandatory for benefits on glucose tolerance in an over-night perspective. It was further suggested that the benefits on glucose regulation was related to gut fermentation of DF[63, 64].

Gut microbiota metabolism in relation to host systemic effects

The distal gut of humans harbors a highly dense microbial ecosystem comprising up to 10^{12} organisms per ml of luminal content[65]. The gut microbiota is referred to as an

"organ", due to the mutualistic relation between the host (e.g. the human) and the microbes, including e.g. exchange of nutrients and metabolites[66]. It has been reported that the composition of the gut microbiota in obesity and T2D differs compared to that of "healthy" counterparts[67, 68], but also differentiates between younger (< 46 years) and older (> 65 years) adults[69]. Also, variation of gut microbiota composition at a population-based level has been suggested, distinguishing three major clusters or so-called "enterotypes", dominated by *Bacteroides*, *Prevotella*, and *Ruminococcus*, respectively[70]. However others detected only *Prevotella* and *Bacteroides*[71]. On the other hand, when discriminating between a less ("low gene count") and more rich ("high gene count") microbiota, significant associations were observed between a less rich microbiota and the dominance of *Bacteroides* and *Ruminococcus* whereas *Bifidobacterium*, *Lactobacillus* and *Akkermansia* were associated with a richer microbiota[72]. Also a less diverse microbiota was associated with metabolic disturbances including characteristics such as more marked overall adiposity and insulin resistance, and a higher inflammatory tonus[72]. However, a 6-week energy restricted dietary intervention including protein, low-GI carbohydrates and supplemented with soluble fibres, increased microbiome richness among the less diverse microbiota subjects, with concomitant benefits on systemic metabolic status[73]. In accordance, the prevalence of the different enterotypes was further related to differences in long-term dietary patterns, implicating a Western diet, including animal protein, and saturated fat as promoting the *Bacteroides* and a carbohydrate-based diet promoting *Prevotella* enterotypes[71]. Further, short-term dietary interventions (10 days) including either a high-fat/low-fibre or low-fat/high fibre diet, induced rapid and significant changes in gut microbiome composition, however, not sufficient to switch individuals between enterotype clustering[71]. Also among elderly, a high diversity of the gut microbiota was promoted by a low-fat/high-fibre diet with concomitant associations with improved health status[74]. Consequently the gut microbiota composition and diversity appears to be affected by diet with potential to promote health benefits, also in a short time perspective, such as over 10 day. Information from long-term interventions with carbohydrate-based diets rich in e.g. fruits and vegetables reports promoted gut microbiota diversity and composition, also associated with health benefits in both young and old adults[71, 74]. The mechanisms whereby gut microbiota executes health promoting effects are not fully elucidated. It is established though that the presence of bacteria in the intestine is important in relation to certain biological aspects of the host, e.g. the

metabolism and energy-harvesting from indigestible carbohydrates, the differentiation of the intestinal epithelium and the involvement in the host immune system[75].

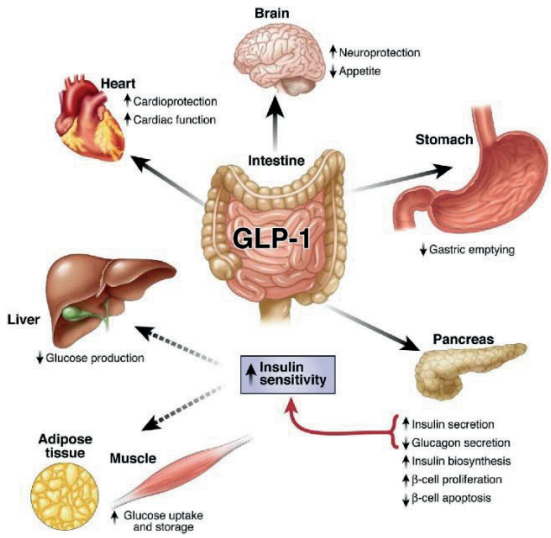

Figure 1. GLP-1 actions in peripheral tissues.
Reprinted from Gastroenterology, 132/6, Baggio L. L., Drucker D. J., Biology of Incretins: GLP-1 and GIP, p 2131-57, 2007, with permission from Elsevier.

The gut-brain axis

The "gut-brain axis" refers to the fact that nutrients or metabolites from digestion of food activate specific receptors located at the luminal surface of enteroendocrine cells, e.g. L-cells, with potential effects on hormone systems involved in e.g. glucose homeostasis[76], appetite regulation and neurosignaling[77, 78], and even regulation of fat deposition in adipocytes[3]. Specific gut hormones are thus released into the circulation targeting e.g. neuronal sites involved in appetite regulation directly in the brain or via the vagus nerve[78]. Glucagon-like peptide (GLP)-1, oxyntomodulin (OXM) and peptide YY (PYY) are examples of hormones released from the L-cell in response to intestinal stimuli and considered as "satiety" hormones involved in appetite regulation[79, 80]. Recently, also GLP-2, a L-cell produced hormone, was shown to promote satiety by activation of specific appetite-regulating regions in the brain[81]. Another relevant hormone involved in gut-brain neuroendocrine signaling includes the peptide ghrelin, originating from the upper-gut and involved in the regulation of hunger[82]. The metabolic actions of the mentioned

hormones are often manifold. Thus, e.g. GLP-1 possess additional important properties such as stimulation of pancreatic insulin secretion in response to an oral glucose load (the "incretin effect") and is thereby involved in regulating postprandial glycaemia[83]. The different actions of GLP-1 are illustrated in Figure 1.

Consistent with the involvement in blood glucose- and appetite regulation, GLP-1 has been ascribed both anti-diabetic and anti-obesity features and pharmacological treatments based on the actions of GLP-1 are available for patients with T2D[84]. However, augmentation of endogenous GLP-1 release has been observed by specific nutrients such as DF[85] and specific proteins[86]. Nutrient stimulation of specific endogenous hormones important to metabolic control, such as e.g. GLP-1 and GLP-2, represent an exciting approach in the prevention of metabolic disorders[87].

Gut fermentation of indigestible carbohydrates

A mechanism whereby gut hormones may be stimulated relates to gut microbiota fermentation of indigestible carbohydrates. This process yields energy for the microbiota and produces metabolites such as short-chain fatty acids (SCFA; mainly acetate, propionate and butyrate) and gases (i.e. H_2 and CO_2) as waste products[88]. The SCFA are absorbed by the host and promotes benefits for host metabolism in terms of acting as energy substrates (e.g. butyrate for intestinal epithelial cells[89]) and as circulating signaling molecules involved in various processes of metabolic relevance e.g. lipid metabolism, gastrointestinal tract functions and inflammation[35, 36]. A shift in the gut microflora towards increased levels of bifidobacteria, mediated by increased intake of certain fermentable DF (e.g. fructo-oligosaccharides) beneficially influence glucose tolerance in mice[90]. The mechanisms behind the improved glucose regulation are still largely unknown, but a connection between changes in intestinal microbiota and effects of GLP-1 has been put forward[90]. It has been observed (*in vitro*) that receptors for SCFA are located in colonic enteroendocrine cells, e.g. L-cells, in rats[91] and humans[92, 93] and they might function as a sensor for luminal SCFA. Further, it has been shown that SCFA trigger the secretion of GLP-1 (colonic cultures), and mice lacking receptors for SCFA experiences reduced SCFA-triggered GLP-1 secretion (*in vivo*), with a concomitant decrease in glucose tolerance[94]. In healthy humans, supplementation of fermentable DF (fructan) during two weeks demonstrated increased levels of endogenous GLP-1, increased markers of colonic fermentative activity (breath H_2) as well as decreased rates of

hunger[95]. The results suggest a link between the intestinal environment, i.e. the action of gut microbiota fermentation, and the metabolic response of the host.

Metabolic endotoxemia

Recently, the term metabolic endotoxemia has been introduced, based on studies in animal models predictive of the human MetS. Metabolic endotoxemia, represents a state where a high-fat diet promotes lipopolysasaccharide (LPS)-containing microbiota in the gut, with a concomitant increase in plasma LPS concentrations[96]. LPS are structural components of the outer membrane of Gram-negative bacteria and are known to activate inflammatory signaling pathways with concomitant release of pro-inflammatory cytokines[97]. With respect to the tight connection between low-grade inflammation and metabolic disorders, it could be hypothesized that the pro-inflammatory LPS may act as a key molecule involved in early development of low-grade systemic inflammation[98]. Increased concentrations of circulating LPS have been strongly associated with components of the MetS[99] indicating a role of metabolic endotoxemia in the etiology of T2D and CVD. Also, subcutaneous infusions of LPS in mice to mimic high-fat diet induced metabolic endotoxemia, resulted in similar metabolic derangement as the high-fat feeding, e.g. increased body weight and impaired blood glucose tolerance[96].

Intestinal permeability and indigestible carbohydrates

The influx of pro-inflammatory LPS to the circulation has been suggested to be mediated, at least in part, by changes in intestinal permeability. The intestinal epithelium acts as a barrier against translocation of potential harmful substances. A decline in the protective function, i.e. increased permeability of the intestinal epithelium has been observed both in genetically or diet-induced obese mice[100, 101], but also among obese humans[102]. In humans the concentration of circulating LPS increases within a few hours after consumption of a high-fat meal, and it has been observed that the baseline and the postprandial increase of LPS is significantly higher among T2D and obese subjects compared to healthy normal weight controls[103]. It has been concluded based on data from animal models, that altered intestinal permeability is correlated with obesity, however a causal relationship is not established[104].

Interestingly, levels of metabolic endotoxemia in diet-induced or genetically obese mice were normalized when supplementing the high-fat diet with fermentative carbohydrates

(oligofructose), with concomitant benefits on glucose metabolism[90, 105]. Recently Lecerf *et al.* (2012) observed decreased plasma LPS in healthy humans after 4 weeks supplementation of the subjects normal diet with a mixture of indigestible carbohydrates (xylo-oligosaccharide and inulin) compared with a placebo supplement[106]. Additionally, both subjects and mice that received dietary supplementation of indigestible carbohydrates also displayed changes in gut microbiota composition and improved inflammatory status[105, 106]. GLP-2 is a gut derived hormone important to intestinal barrier functions[107]. Concentrations of GLP-2 have been found to increase by gut microbiota changes due to prebiotic (oligofructose) treatment in mice, with a concomitant improvement of gut barrier functions and decreased inflammation[108]. Exposure of butyrate to colon-derived epithelial cell lines has been shown to reduce metabolic stress and decrease epithelial permeability, indicating the relevance of the epithelial barrier function[109].

Taken together, the results highlight a role of indigestible substrates and the gut microbiota composition in the etiology of systemic low-grade inflammation in relation to MetS-associated diseases.

Modulation of gut microbiota by diet and/or dietary supplements

The concept of prebiotics was originally described by Gibson *et. al.* (1995) and defined as "a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health"[110]. Today, only a few substrates have met the criteria for prebiotics, in the meaning of having shown to resist digestion in the human small intestine, reach the colon and act as fermentation substrate by the gut microflora, with observed stimulation of the specific bacterial genus *Bifidobacterium*[111, 112].

Also other sources of indigestible carbohydrates than those stimulating bifidobacteria could be considered as potentially health promoting by influencing the metabolism of the gut microbiota. The prebiotic potential of foods rich in indigestible carbohydrates is thus an interesting topic.

Other means to modulate the human colonic microbiota is through probiotics. A probiotic is defined as "live microorganisms that, when administered in adequate amounts, confer health benefit on the host"[113]. In a recent consensus report it was

suggested that effects of specific probiotic strains could be mediated by mechanisms related to intestinal barrier functions, interactions with immune intestinal cells, modulation of endogenous gut microbiota and suppression of potential intestinal pathogens[114]. However, conclusions regarding associations between consumption of probiotics and management of metabolic disorders such as obesity and diabetes could not be done due to limited number of human studies[114]. However, recent studies indicate that probiotic supplementation with *Akkermansia muciniphila* during 4 weeks to mice normalized diet-induced metabolic endotoxemia, reduced body weight, restored gut barrier function, and completely reversed diet-induced fasting hyperglycaemia[115]. Supplementation of *Lactobacillus plantarum*, in a subset of mice in the same study, did not induce any metabolic benefits as were seen with *A. muciniphila*.

Health aspects of whole grain and legumes

Both WG cereals and legumes (e.g. *Phaseolus vulgaris*) are rich sources of DF and represents indigenous foods historically included in the human diet. However, even though several health benefits have been related to DF intake[17], the current daily intake of DF is low. The recommended intake of DF in adults corresponds to 25-38 g/day in the U.S.[116], which is similar to the recommendation in the Nordic countries[117]. The actual intake though corresponds to a much lower figure, approximately 16 g/day in the U.S.[118] and somewhat higher, or 20-22 g/day in Sweden[119]. Estimating the source of DF, approximately 40-50% of the daily DF intake origins from WG cereal products[116, 119]. Traditionally legumes have been an important part of the diet in many cultures. However, the use of legumes in developed countries today is inferior. Legumes, i.e. peas, beans, lentils, peanuts and other podded plants, are apart from being low-GI foods and good sources of DF also generally rich in dietary protein with high biological value, and desired low amounts of saturated fat[120].

Diets rich in DF have been associated with health-promoting benefits in relation to MetS-associated diseases. For instance, it has been observed that consumption of WG bread and cereal DF were related to decreased risk of T2D[18] and cross-sectional studies indicates that high intake of WG is associated with low prevalence of overweight and obesity[21, 23]. In addition, a reduced risk of being obese was observed among bean consumers when compared to non-consumers[121]. Further, DF are suggested to influence

appetite control by promoting satiety and reducing energy intake. However, different types of DF, or whether the DF is consumed as an isolated fibre supplement or naturally occurring in food may differently affect satiety. For example, specific viscous soluble fibre, e.g. β-glucans, have been observed to increase satiety[122, 123].

Several aspects of the favorable metabolic outcomes of DF-rich diets have been discussed, such as e.g. low GI features, DF per se and/or the presence of DF associated bioactive components, such as lignans, tocotrienols, phenolic compounds, phytic acid, tannins and minerals[53, 54, 124-126]. Different mechanisms have been suggested in regards of the appetite regulatory properties of DF-rich foods and involves effects on gastric distension (e.g. by water binding capacity of viscous fibre), reduced gastric emptying rate and through activation of gut-derived hormones[112].

To conclude, foods rich in specific fermentable indigestible carbohydrates may play an important role in relation to their potential preventive value adjunct to disorders related to the MetS.

Definitions of WG and DF

The definition of WG differs slightly between different parts of the world. The definition taken by HEALTHGRAIN (EU 6th Framework Programme Integrated Project "*Exploiting Bioactivity of European Cereal Grains for Improved Nutrition and Health Benefits*"), was that "whole grains shall consist of the intact, ground, cracked or flaked kernel after the removal of inedible parts such as the hull and husk. The principal anatomical components - the starchy endosperm, germ and bran - are present in the same relative proportions as they exist in the intact kernel"[127]. Thus, the definition of WG is applied to both milled cereal flours (whole meal) and kernels. The germ and bran, typically removed from refined cereal products, consists of several bioactive compounds such as vitamins, minerals, lignans and phenolic acids, but are also rich sources of indigestible carbohydrates including NSP, RS and oligosaccharides[54]. The proportion and composition of DF vary between different cereals such as wheat, rye, oat and barley[54]. The content of RS may differ depending on food processing conditions[128, 129]. Barley and oats are naturally rich in β-glucan with suggested health-promoting effects[130]. Accordingly, The European Food Safety Authority, Panel on Dietetic Products, Nutrition and Allergies, established a cause and effect relationship between consumption of barley-

or oat β-glucans and a reduction of postprandial glycaemic responses and the maintenance of normal blood cholesterol levels[131].

Several definitions of DF exists. However, the current Codex Alimentarius definition is: "Carbohydrate polymers with ten or more monomeric units, which are not hydrolyzed by the endogenous enzymes in the small intestine of humans"[132]. In addition, also RS and oligosaccharides with a degree of polymerization (DP) ≥ 3 are considered DF constituents. However, it has been stated that it is up to national authorities whether to include oligosaccharides (DP of 3-9) in the definition, which has been done e.g. by the European Commission and the American Association of Cereal Chemists[133, 134].

Objective

The overall objective of this thesis was to investigate barley- and legume products with maintained botanical integrity and rich in intrinsic DF-components, with respect to their ability to influence risk markers related to the MetS. In particular, the possible link between gut fermentation of DF-components and metabolic responses was evaluated with focus on the involvement of gut hormones.

For this purpose, four studies were performed in an "over-night" perspective, addressing the metabolic effects of barley kernel products and legumes with emphasis on blood glucose regulation, inflammatory markers and appetite regulation. Three studies included normal-weight young adults, and one study middle-aged, normal to slightly overweight subjects. The reference food (white wheat bread) or test foods were consumed as late evening meals, or during three or four consecutive days prior to the subsequent experimental sampling in the morning of the study day. All metabolic measurements were performed after an over-night fast (approximately 10 h). On the study day, subjects were provided standardized breakfast meals and markers of metabolism and appetite regulation were analyzed as well as markers of gut fermentative activity, i.e. breath H_2 and circulating SCFA. In addition to subjective rating of appetite sensation, two studies also included measurement of voluntary food intake on the study day.

Material and Methods

An overview of the studies, test products, subjects, interventions and test variables are summarized in Table 5 (page 45).

Test- and reference products

Barley kernel products

In Papers I, II and IV commercially available barley kernels (*Hordeum vulgare* L.) were used. It was a blend of non-specified Swedish varieties that were slightly polished and dried (Finax, Helsingborg, Sweden). In Paper I the barley kernels (BK) were served cooked with the appearance of a rice-analogue and the test subject prepared and consumed the specified portion in their home according to instructions. The test product was consumed with 250-300 ml of water.

In Papers II and IV, the barley kernels were baked into bread (BB) according to instructions presented in each paper. The proportion of cereal based ingredients expressed as dry matter (dm) in the BB of Paper II was whole barley kernels (85% of dm) and white wheat flour (15% of dm). The BB of Paper IV was kindly produced by Credin A/S (Juelsminde, Denmark) and contained whole barley kernels (75% of the dm) blended with a minor part of whole-grain barley flour (10% of the dm) and wheat flour (15% of the dm).

Supplementation of established probiotics and non-probiotic control

In Paper IV a mixture of commercially available probiotics was included. The dosage of each probiotic corresponded to the recommended daily dose according to the manufacturer. The selection of the probiotics were based on the availability of the

products to the Swedish consumer. The selected strains are considered common on the Swedish market. The following three different strains and doses were included.

- *Bifidobacterium animalis* DN-173 010 (ACTIREGULARIS®), $20 \cdot 10^9$ CFU/day, (Activia®, Danone AB, Solna, Sweden).
- *Lactobacillus reuteri* DSM 17938 (Protectis®), $10 \cdot 10^9$ CFU/day (PROBIOMAX®, BioGaia AB, Stockholm, Sweden)
- *Lactobacillus plantarum* 299v, $0.1 \cdot 10^9$ CFU/day (ProbiMage®, Probi AB, Lund, Sweden)

The first probiotic was served as a yoghurt product, and the second and third were administered as tablets. Conventional yoghurt (Skånemejerier, Malmö, Sweden) and maltodextrin were included as a non-probiotic control.

Legumes

Paper III included common beans (*Phaseolus vulgaris* L.) of different varieties (red "kidney", white "Cannellini", brown or black) and chickpeas (*Cicer arietinum*). The beans were soaked in water for 12 h before being boiled and consumed directly after preparation. The test subjects prepared and consumed the beans in their home according to instructions. Water, at optional amount was consumed with the beans, however the subjects were obliged to maintain the same amount of water throughout the study.

WWB

In Papers I-IV a low DF, high GI white wheat bread was used as reference. In Papers I-III the WWB was baked according to a standardized procedure in a home baking machine. Papers I and III: Severin model nr. BM 3983; Menu choice, program 2 [white bread, 1000 g, quick (time 2:35)] and Paper II: Tefal home bread model nr. 573102; Menu choice, program 2 [white bread, 1000 g, quick (time 2:32)]. The bread was made from 540 g of white wheat flour (Kungsörnen AB, Järna, Sweden), 360 g water, 4.8 g dry yeast, 4.8 g NaCl. After cooling to ambient temperature, the bread was sliced and wrapped in aluminum foil in portions sizes, put into plastic bags and stored in a freezer (-20ºC). In Paper I and III the crust was removed before slicing and packaging. In Paper IV, a commercial WWB (Dollar Storfranska, Lockarp, Malmö, Sweden) was included. The

procedure with respect to packaging and storage was the same as described for the bread in Papers I-III. The test subjects received the bread frozen to be put in their freezer at home without any delay. At the day of consumption the subjects were instructed to thaw the bread at ambient temperature, still wrapped in aluminum foil and maintained in the plastic bag.

Supplementation of non-probiotic control

The WWB in Paper IV was provided in combination with conventional yoghurt (Skånemejerier, Malmö, Sweden) and maltodextrin and served as a non-probiotic control.

Servings

In all four papers the amount of test- and reference product to be consumed was based on the amount of available starch, further explained below (*Chemical analysis of the test- and reference products and meals*). In Paper I, the portion size of the evening test- and reference meals was 50 g available starch. Due to the bulkiness of beans the portion sizes in Paper III were decreased to allow for a realistic meal size. Thus the portion size of those test- and reference meals was set in provide 35 g available starch. In Papers II and IV the test- and reference products were included in the diet for three or four days, respectively. In Paper II, the quantity of the test- and reference products to be ingested daily was standardized to provide 100 g of available starch per day. The daily intake of test- or reference product was divided into three equal portions to be consumed at approximately 0800, 1400 and 2100 h for the first two days. On the third day, half of the daily intake (50 g available starch) was divided equally between the 0800- and 1400 h meals, and the other half (i.e. 50 g available starch) was consumed at 2100 h in the evening. In Paper IV, the intervention time was extended to four consecutive days and the daily intake of the test- and reference products was adjusted to provide 75 g available starch. The daily portions were divided into two equal portions to be consumed in the morning and the evening during the first three days, and on day four the morning portion provided 25 g available starch and the late evening portion corresponded to 50 g available starch.

Standardized meals

Standardized breakfast

In Papers II, III and IV a standardized WWB portion based on 50 g available starch was served as breakfast. In Paper III a commercially available bread was served (Dollar Storfranska, Lockarp, Malmö, Sweden) and in Papers II and IV the standardized breakfast consisted of a WWB baked according to previously described standardized procedure in a home baking machine[135]. The breakfast was served with a fixed amount of tap water (200 ml, Paper IV; or 250 ml, Papers II and III).

Ad libitum breakfast

In Paper I, an ad libitum breakfast was included. The breakfast consisted of commercially available WWB (Dollar Storfranska, Lockarp, Malmö, Sweden) with butter (Bregott, Arla Foods, Stockholm, Sweden) and ham. The sandwiches were cut in small pieces (6.5 x 6.5 cm) and served as double-sandwiches whole or cut diagonally. The subjects were allowed to choose freely the amount of sandwiches to consume and the quantities were registered. The breakfast was served with a predetermined portion of 300 ml tap water.

Ad libitum lunch

In Papers I and IV an ad libitum lunch was served at 210 min after commencing the breakfast. The lunch consisted of Swedish hash i.e. fried mix of diced potato, meat and onions (Felix Krögarpytt, Procordia Food AB, Eslöv, Sweden). The test subjects could choose freely the amount to be consumed and the quantities were registered. In Paper I the test subjects were allowed to add ketchup (Felix, Procordia Food AB, Eslöv, Sweden) to the lunch. If ketchup was chosen to be consumed with the hash, the subject was obliged to maintain same amount of ketchup throughout the study. The lunch was served with 250 ml (Paper I) or 200 ml (Paper IV) tap water.

Chemical analysis of the test- and reference products and meals

The contents of starch and DF in the test- and reference products are presented in Table 2 (page 43). The content and distribution in test products is illustrated in Figure 3 (page 46).

The present thesis distinguishes the following DF constituents, soluble- and insoluble NSP, RS and oligosaccharides, which are analyzed separately and included in the total DF. It should be noted that the total DF in the published Paper I includes only NSP in the DF, and RS is reported separately. However, total DF content in the published Paper III refers to the sum of NSP, RS and oligosaccharides according to the most recent definition as referred to above.

The composite breakfast and lunch meals in Paper I were analyzed for available starch, protein and fat, respectively, in order to calculate total energy intake. Energy content of the ad libitum lunch meal in Paper IV was calculated according to the nutritional composition provided by the manufacturer. The nutritional composition of the breakfast and lunch meals in Paper I is presented in Table 3 (page 37).

Total, available and resistant starch

Test- and reference products

The test- and reference products were analyzed with respect to total starch[136], available starch[137] and RS[138]. Prior to analysis of total- and available starch as well as DF, the products were air dried and milled (Cyclotec, Foss Tecator AB, Höganäs, Sweden). The BK (Paper I) and the brown beans (Paper III) were boiled prior to drying. RS was analyzed on products as eaten.

Available starch content of the test products in all papers, as well as the reference WWB product in Papers II and III, was calculated by subtracting RS from total starch. However available starch of reference WWB in Papers I, and IV was determined according to Holm *et. al* (1986)[137]. Final portion sizes of test- and reference products were based on analyzed starch contents with methods described in respective Paper (I-IV).

Breakfasts and lunch (Paper I)

The ad libitum breakfast sandwiches in Paper I were prepared as eaten and then cut into small pieces and freeze dried prior to analysis. The lunch was prepared according to the manufacturers' instructions and then mixed with water into a paste, followed by freeze drying. Freeze dried samples of the breakfast sandwiches and the lunch were ground in a mortar prior to analysis.

The available starch content of the breakfasts in Paper I, II and III were determined according to Holm *et al.* (1986)[137] and in Paper IV by subtracting RS[138] from total starch[136]. The ad libitum lunch meal in Paper I was analyzed with respect to available starch[137].

Dietary fibre

Insoluble- and soluble DF were, in all studies, determined with a gravimetric, enzymatic method on air dried and milled samples[139] of test- and reference products prepared as eaten.

Raffinose

In Paper III, the legume test products and the reference WWB were analyzed for quantification of raffinose (Kit Raffinose/Galactose K-RAFGA 07/11, Megazyme International Ireland Ltd). However, the α-galactosidase in the kit kit did not only hydrolyses raffinose but also other α-galactosides, such as stachyose and verbascose.

Protein

Crude protein content of the breakfast- and lunch meals in Paper I was determined using an elemental analyzer (FlashEA 1112, Thermo Fisher Scientific Inc, Waltham, MA, USA).

Fat

Fat content of the breakfast- and lunch meals in Paper I was measured gravimetrically using the Schmid-Bondzynski-Ratzlaff (SBR) method.

Table 3. Nutritional composition of the breakfast and lunch in Paper I[1]

	Carbohydrates[2]	Protein	Fat	Energy content
		% dry matter		kcal
Breakfast	62.6	11.9	13.6	59.8 /sandwich
Lunch; "Hash"	38.5	13.9	27.5	234 /100 g
Ketchup[3]	-	-	-	85/100 g

[1] The breakfast consisted of commercial white wheat bread with butter and ham and the lunch consisted of Swedish hash, composed of a mix of fried diced potato, meat and onion with the voluntary addition of ketchup. Values of carbohydrate and fat are based on means of 2 replicates, protein means of 4 replicates.
[2] Analyzed as available starch according to Holm *et. al* (1986)[137].
[3] Values obtained from the manufacturer.

Meal studies

Test subjects

In Papers I, III and IV young, healthy test subjects (aged 23.4 ± 0.4 years; BMI 22.4 ± 0.4 kg/m^2) were included whereas Paper II included middle-aged, healthy subjects (aged 64.1 ± 1.3 years; BMI 23.6 ± 0.5 kg/m^2). A total of 56 young volunteers (25 men and 31 women) participated in Papers I, III, and IV and in Paper II, 20 (3 men and 17 women) subjects were included. All subjects had normal fasting blood glucose concentrations (≤ 6.1 mmol/L) and were non-smokers (or non-snuff users), overall healthy and without known metabolic disorders or food allergies. Anti-hypertensive medications (Paper II, 1 subject) and prescription-free painkillers without anti-inflammatory action were accepted. Written informed consent was obtained from each subject. All studies were approved by the Regional Ethical Review Board in Lund, Sweden. Papers II, III and IV were registered at ClinicalTrials.gov (II: NCT01718431; III: NCT01706042; IV: NCT01718418).

Study design

A randomized cross-over design was used in all four studies. However, in Paper IV the intervention products BB(-) and WWB(-) were randomized but the third intervention product BB(+) was in all cases studied at last, in order to avoid potential remaining effect of the probiotics. In Papers I and III the test- and reference products were included as late evening meals (0900-0930 pm) prior to the experimental day. In Paper II the test- and reference products were consumed during three consecutive days and in Paper IV the test- and reference products were consumed during four consecutive days prior to the experimental day. In all studies, fasting and postprandial measurements were performed just before and following a high-GI breakfast based on WWB, and in Paper I also after a lunch meal. The time interval at the experimental day differed between the studies and was: 0-330 min (Paper I), 0-240 min (Paper IV), 0-180 min (Paper III) and 0-150 min (Paper II). Time point 0 min indicates the initiation of the breakfast. A schematic overview of the study design is illustrated in Figure 2.

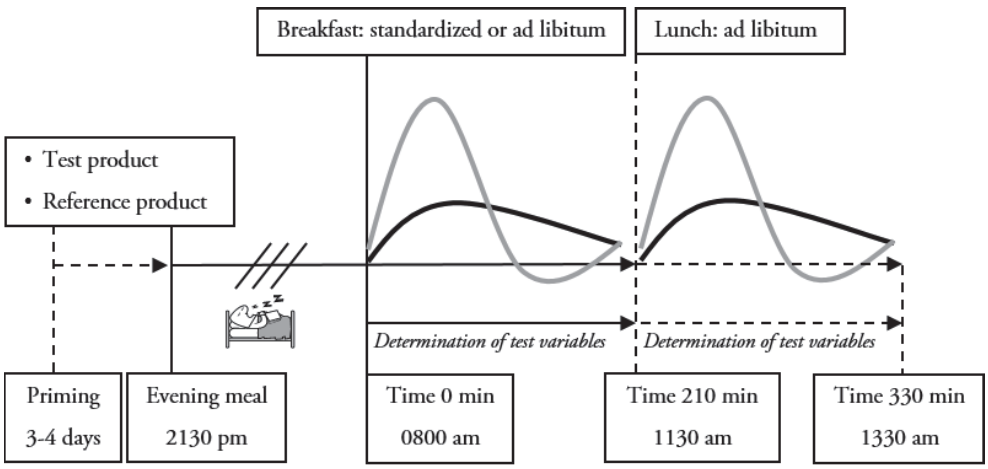

Figure 2. Schematic overview of the experimental design used in the meal studies presented in this thesis.
Randomized cross-over designs with some differences between studies were used in Paper I-IV, respectively. WWB, white wheat bread.

Procedure

The subjects were encouraged to standardize their meal pattern and otherwise maintain their regular eating habits during the experimental periods. They were also instructed to avoid alcohol, excessive physical exercise or food rich in DF the day prior to the evening

test- or reference meals (Papers I and III) or during the entire intervention periods (Paper II and IV). Furthermore, they should not have taken antibiotics or probiotics during 2 weeks prior to the study and throughout the duration of the study. The test subjects prepared and consumed the test- and reference products in their home according to detailed instructions with respect to preparation of the meal, amount of water and time schedule for initiation and finishing the meal. After the evening meals, the subjects were fasting until the breakfast was served the subsequent morning at the research department. The subjects arrived at ~0730 am. An intravenous cannula (BD Venflon, Becton Dickinson) was inserted into an antecubital vein for blood sampling. Blood samples were collected and appetite and breath H_2 registered before the breakfast and continued repeatedly after the breakfast. The breakfast was consumed at approximately ~0800 am and finished within 15 min. In Paper I, the test subjects were offered coffee or tea (without milk or sugar) or water (200 ml) at 120 min after the breakfast, each subject keeping the same drink throughout the study. During the experimental days the subjects were told to maintain a constant, low physical activity, preferable reading or computer work.

Physiological variables and subjective appetite rating

A detailed time schedule regarding sampling of physiological variables and subjective appetite ratings are summarized in Table 4 (page 44).

Blood glucose

Finger-prick capillary blood samples were taken for determination of blood (b-) glucose and analyzed using HemoCue® B-glucose (HemoCue AB, Ängelholm, Sweden).

Plasma and serum insulin

Insulin was determined at fasting and in postprandial blood in all four studies. In Papers II-IV serum (s-) insulin was determined with a solid phase two-site enzyme immunoassay kit (Insulin ELISA 10-1113-01, Mercodia AB, Uppsala, Sweden). In Paper II, the analysis was executed using an integrated immunoassay analyzer (CODA Open Microplate System; Bio-rad Laboratories, Hercules, CA, USA). In Paper I, plasma (p-) insulin was analyzed with Milliplex™ MAP (HMH-34K Milliplex™ MAP, Millipore, St.Charles, USA). The latter analysis was performed simultaneously for insulin, active ghrelin, total GIP, and active GLP-1, using immunoassays on the surface of fluorescently

labelled microsphere beads and read on the Luminex 200 instrument (Luminex Corporation, USA). The plasma was collected into tubes containing an inhibition cocktail consisting of DPPIV-inhibitor (10 µl/ml blood) (Millipore, St Charles, USA) and Pefablock SC (1 mg/ml blood) (Roche Diagnostics, Mannheim, Germany). Milliplex™ Analyst v3.4 (VigeneTech Inc., Carlisle, USA) was used for the evaluation of the results.

GLP-1, GIP, GLP-2, PYY and OXM

In Paper I p-GLP-1 (active) was analyzed using the Milliplex™ MAP (HMH-34K Milliplex™ MAP, Millipore, St.Charles, USA) technique as described above for insulin. In Papers II-IV, quantitative determination of active GLP-1 (7-36) was performed in plasma collected in tubes prepared with an inhibition cocktail consisting of DPPIV inhibitor (10 µl/ml blood) (Millipore, St Charles, USA) and Trasylol® 10 000 KIE/ml Aprotinin (50 µl/ml blood) (Bayer HealthCare AG, Leverkusen, Germany). The analysis was performed with a highly sensitive ELISA kit (GLP-1 (active 7-36) ELISA 43-GP1HU-E01 ALPCO Diagnostics, Salem, NH). Total p-GIP concentrations in Paper I was analyzed using the Milliplex™ MAP technique described above. In Papers II-IV, the concentration of p-GLP-2 was determined with a competitive enzyme immunoassay (Human GLP-2 EIA YK141, Yanaihara Institute Inc. Shizuoka, Japan). The plasma was collected in tubes prepared with an inhibition cocktail as described for the ELISA analysis of GLP-1. In Papers II and IV, p-PYY (both PYY (3-36) and PYY (1-36)) concentrations were determined with a competitive enzyme immunoassay (Human PYY EIA YK080, Yanaihara Institute Inc. Shizuoka, Japan). The plasma was collected in tubes prepared with an inhibition cocktail as described for the ELISA analysis of GLP-1. In Papers II and III, p-OXM was determined in Plasma collected in tubes prepared with an inhibition cocktail as described for the ELISA analysis of GLP-1. The analysis were performed with commercial kits (Paper II; Human Oxyntomodulin ELISA kit CSB-E12948h, Cusabio biotech CO., Ltd, Wuhan, P.R China, and paper III; human Oxyntomodulin ELISA Kit XSB-E12948h, Cusabio Biotech CO., Ltd, Newark, USA).

Ghrelin

In Paper I, active p-ghrelin was determined using the Milliplex™ MAP technique described above. In Paper II and IV p-ghrelin was analyzed with an enzyme immunometric assay (Human Acylated Ghrelin ELISA RD194062400R, BioVender GmbH, Heidelberg, Germany). The plasma was collected in tubes prepared with an

inhibition cocktail as described for the ELISA analysis of GLP-1. Additionally the plasma intended for ghrelin-analysis in Paper II and IV was treated with 1 M HCl prior to freezing (10:1).

Glucagon

In Paper IV, p-glucagon was determined collected in tubes prepared with an inhibition cocktail as described for the ELISA analysis of GLP-1. A solid-phase immunoassay (Quantikine® ELISA; Glucagon Immunoassay DGCG0, R&D Systems, Abingdon, UK) was used.

IL-6, IL-18, CRP and PAI-1

Enzyme immunoassays were used to determine the concentration of s-IL-6 in Papers I, II and IV and s-IL-18 concentrations in Papers II and IV (Quantikine® HS ELISA, Human IL-6 HighSensitive HS600B, R&D Systems, Abingdon, UK, and Human IL-18 ELISA Kit 7620, MBL Medical & Biological Laboratories CO., Ltd, Nagoya, Japan). In Paper IV, s-CRP and s-PAI-1 were analyzed using commercially available ELISA kits (CRP ELISA Kit, Immundiagnostik AG, Bensheim, Germany and Human PAI-1 ELISA, RBMS2033R, BioVendor GmbH, Heidelberg, Germany, respectively).

Adiponectin

In Papers I and III, adiponectin was determined in plasma and in Papers II and IV in serum. In all studies a solid phase two-site enzyme immunoassay kit (Mercodia Adiponectin ELISA 10-1193-01, Mercodia AB, Uppsala, Sweden) was used.

FFA and Triglycerides

In Papers I-IV, s-FFA concentrations were determined with an enzymatic colorimetric method (NEFA, ACS-ACOD method, Wako Chemicals GmbH, Neuss, Germany). In Paper IV, triglycerides (TG) were determined in serum using a multi-sample enzymatic assay (LabAssay™ Triglyceride 290-63701, GPO·DAOS method, Wako Chemicals GmbH, Neuss, Germany).

SCFA

In Papers I-III, the SCFAs acetate, propionate and butyrate were analyzed, and in Paper III also isobutyrate. SCFA was analyzed in serum in Paper I and II, whereas plasma was

used in Paper III. The determination of SCFA concentration in serum or plasma was performed using a gas-chromatography method[140].

Breath H_2

H_2 in expired air was measured as an indicator of colonic fermentation using a hand-held monitor in all four papers (EC60/Gastro$^+$ Gastrolyzer, Bedfont Scientific Ltd., Rochester, England).

Subjective appetite rating

In all papers the test subjects rated their feeling of satiety, hunger and desire to eat using a 100 mm bipolar visual analogue scale (VAS) graded from "none" to "extreme"[141].

Table 2. Available and indigestible carbohydrates in the test- and reference meals[1]

Products (Paper no.)	Starch		NSP			Total DF
	Available starch	RS	Insoluble DF	Soluble DF	Raffinose[2]	
Composition			*% dry matter*			
BK (I)	57.2	11.6	8.1	5.8	-	25.5
BB (II)	63.5	10.8	8.8	4.2	-	23.8
BB (IV)	59.7	8.0	8.5	4.0	-	20.4
BrB (III)	41.3	7.6	15.4	9.4	3.2	35.7
WWB (I-IV)[3]	77.3 ± 0.8	2.1 ± 0.2	3.5 ± 0.3	1.5 ± 0.5	0.1	6.6 ± 0.3
Portion size			*g/evening meal*			
BK (I)	50	10.1	7.1	5.1	-	22.3
BB (II)	50	8.5	7.0	3.3	-	18.8
BB (IV)	50	6.7	7.1	3.3	-	17.1
WWB (I, II, IV)[3]	50	1.2 ± 0.04	2.2 ± 0.16	1.1 ± 0.37	-	3.7 ± 0.25
BrB (III)	35	6.5	13.5	8.0	3.0	31.0
WWB (III)	35	1.2	1.8	0.3	0.1	3.36

[1]Values of available starch are based on means of 2 replicates, RS means of 6 replicates, insoluble and soluble NSP means of 3 replicates, raffinose means of 2 replicates. Available starch was calculated by subtracting RS from total starch, except for the WWB in Papers I and IV were available starch was analyzed according to Holm *et al.* (1986)[137]. Included in total DF are RS analyzed as described by Åkerberg *et al.* (1998)[138], insoluble- and soluble NSP determined gravimetrically according to Asp *et al.* (1983)[139] and raffinose, see *Chemical analysis of the test- and reference products and meals*. (-) indicates that no analysis has been performed. BB, barley kernel based bread; BK, boiled barley kernels; BrB, boiled brown beans; DF, dietary fibre; NSP, non-starch polysaccharide; RS, resistant starch; WWB, white wheat bread (reference).

[2]The α-galactosidase in the kit used for quantification of raffinose, also hydrolyses other α-galactosides, such as stachyose and verbascose.

[3]Values are presented as means ± SEM based on WWB from the papers within brackets, respectively.

Table 4. Time schedule for sampling of test variables in respective paper.

	0[1]	15	30	45	60	90	120	150	180	210[1]	225	240	255	270	300	330
Glucose	I-IV	I-IV	I-IV	I-IV	I-IV	I-IV	I-IV	II, III	I, III, IV	I, IV	I	I	I	I	I	I
Insulin	I-IV	I, IV	I-IV	I-IV	I-IV	I-IV	I-IV	II	I, IV	I, IV	I	I	I	I	I	I
GLP-1	I-IV	I	I-III	I, II	I-IV	I, II	I, III, IV	II	I, III	I	I	I	I	I	I	I
GLP-2	II, III, IV	IV	II, III, IV	IV	II, III, IV	II, IV	III, IV	II	III	IV						
PYY	II, III, IV		III		II, III, IV		II, III, IV		III	IV						
OXM	II, III		III		II, III		II, III		III							
GIP	I	I	I	I	I	I	I		I	I	I	I	I	I	I	I
Ghrelin	I-IV	I	I-IV	I	I-IV	I, II	I, III, IV	II	I, III	I, IV	I	I	I	I	I	I
Glucagon	IV	IV	IV	IV	IV	IV	IV			IV						
IL-6	I-IV				I-IV		I-IV		III	I, IV				I		I
IL-18	II, III, IV				II, III, IV		II, III, IV		III	IV						
SCFA	I, II, III				I, III		I									
CRP/PAI-1	IV			IV												
TG	IV		IV	IV	IV	IV			IV							
FFA	I-IV							II	III	I, IV						
Adiponektin	I-IV				I, III, IV		I-IV		III, IV	I, IV				I		I
Breath H_2	I-IV	I, II, IV	I-IV	I, II, IV	I-IV	I-IV	I-IV	II	I, III, IV	I, IV	I	I	I	I	I	I
VAS	I-IV	I, III, IV	I-IV	I-IV	I-IV	I-IV	I-IV	II	I, III, IV	I, IV	I	I	I	I	I	I

[1] Time 0 and 210: variables determined immediately prior to breakfast or lunch, respectively.

Table 5. Overview of the studies, subjects and test products, intervention, and test variables included[1].

Paper	Subjects Age / BMI	Test products	Intervention	Test variables
I	24.2 ± 0.4 years 22.3 ± 0.5 kg/m^2 N = 19	Boiled barley kernels (BK) WWB	Evening meal	b-glucose, p-insulin, p-GLP-1, p-GIP, p-ghrelin, s-FFA, p-adiponectin, s-IL-6 Breath H_2, s-SCFA Subjective appetite sensations Voluntary energy intake (breakfast and lunch)
II	64.1 ± 1.3 years 23.6 ±0.5 kg/m^2 N = 20	Barley kernel based bread (BB) WWB	Three days priming + evening meal	b-glucose, s-insulin, p-GLP-1, p-GLP-2, p-PYY, p-ghrelin, p-OXM, s-FFA, s-adiponectin, s-IL-6, s-IL-18 Breath H_2, s-SCFA Subjective appetite sensations
III	23.8 ± 0.7 years 22.5 ± 0.6 kg/m^2 N = 16	Boiled chickpea, white-, kidney-, or black bean Boiled brown bean (BrB) WWB	Evening meal	b-glucose, s-insulin, p-GLP-1, p-GLP-2, p-PYY, p-ghrelin, p-OXM, s-FFA, s-adiponectin s-IL-6, s-IL-18 Breath H_2, p-SCFA Subjective appetite sensations
IV	23.9 ± 0.7 years 22.6 ± 0.4 kg/m^2 N = 21	BB - probiotics (BB(-) BB + probiotics (BB(+) WWB - probiotics (WWB(-)	Four days priming + evening meal	b-glucose, s-insulin, p-GLP-1, p-GLP-2, p-PYY, p-ghrelin, p-glucagon, s-FFA, s-adiponectin, s-IL-6, s-IL-18, s-CRP, s-PAI-1, s-TG Breath H_2, p-SCFA Subjective appetite sensations Voluntary energy intake (lunch)

[1]Values are presented as means ± SEM. BB, barley kernel based bread; BK, boiled barley kernels; BrB, boiled brown beans; WWB, white wheat bread (reference).The analytical methods are described in the Material and Method section of each paper.

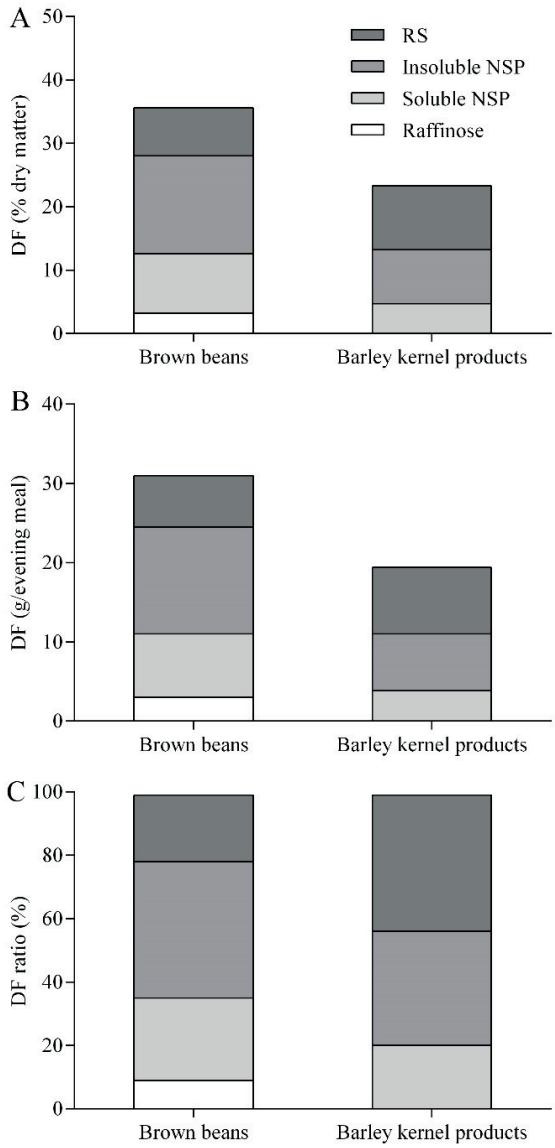

Figure 3. DF content and distribution in barley kernel products and boiled brown beans, respectively. A, DF content and distribution (% dry matter); B, DF content and distribution in an evening meal serving (g). The portion size is calculated to provide 50 g available starch for barley kernel products, and 35 g available starch for brown beans; C, Relative DF distribution (%).

Calculations and statistical methods

The incremental area- and area under the curve (iAUC and AUC, respectively) was calculated for each subject and test- and reference product, using the trapezoid model. Incremental peak (iPeak) concentrations were calculated as individual maximum postprandial increase from baseline. GraphPad Prism (versions 4-6) was used for graph plotting and calculation of iAUC and AUC. For test variables where the variation in the concentration during postprandial measurements did not produce distinct variations over time, a weighted mean was produced by calculating one mean per e.g. hour over the test period, and then an overall mean was calculated and used in statistical analysis. In Paper I, the glycaemic profile (GP^2) was calculated as the time (min) during which the blood glucose remained above fasting concentration divided with the squared incremental peak value of blood glucose for each subject and test- or reference product. In the cases where the blood glucose concentration remained above fasting for the entire 210 min, the duration value was set to 210 min[142]. As a measure of insulin resistance in the morning after each intervention, a homeostatic model assessment, HOMA-IR, were used[143] [fasting glucose (mmol/L) × fasting insulin (nmol/L) × 10^{15}]. For assessment of insulin sensitivity, the composite insulin sensitivity index ($ISI_{composite}$), so called Matsuda index, was calculated including measures of b-glucose and s-/p-insulin in the postprandial phase after the standardized breakfast for test- and reference products, respectively. ($ISI_{composite}$): 10 000/square root of [fasting glucose (mmol/L) × fasting insulin (nmol/L) × glucose iAUC 0-120 min (mmol·min/L) × insulin iAUC 0-120 min (nmol·min/L)][144, 145]. In Papers I, II and III, total SCFA concentration was calculated as the sum of acetate, propionate and butyrate.

Significant differences in test variables after the different test- and reference products were assessed with ANOVA (general linear model (GLM)), in MINITAB Statistical Software (release 14-16; Minitab, Minitab Inc, State College, PA). In the pre-analysis of the legumes in Paper III the ANOVA was followed by Dunnett´s test using reference WWB as control and in Paper IV the ANOVA was followed by Tukey´s pairwise multiple comparison test for means. In the cases of unevenly distributed residuals (tested with Anderson-Darling and considered unevenly distributed when $P < 0.05$), Box Cox transformation were performed on the data prior to the ANOVA, however means are presented as original data. Differences between the products at different time points were evaluated using a mixed model (PROC MIXED in SAS release 9.2 and 9.3; SAS Institute

Inc, Cary, NC) with repeated measures and an autoregressive covariance structure. Correlation analysis was conducted to evaluate the relation among dependent measures with the use of Pearson's correlation in MINITAB Statistical Software (release 14-16; Minitab, Minitab Inc, State College, PA). In Paper IV correlation analysis was performed using Spearman's partial correlation coefficients controlling for subject (two-tailed test, SPSS software, version 22; SPSS Inc., Chicago, IL, USA). Randomization of the test- and reference products was performed in MINITAB Statistical Software (releases 14-16; Minitab, Minitab Inc, State College, PA). Power calculations for determination of number of test subjects were based on experiences of previous over-night meal studies[64] and calculations were performed with MINITAB Statistical Software (releases 14-16; Minitab, Minitab Inc, State College, PA). Primary outcome measure for power calculations was change in blood glucose iAUC (0-120 min).

If the value from one test subject were missing for one of the test- or reference products, the test subject was excluded from that specific calculation. Data are expressed as means ± SEM. *P*-values ≤ 0.05 were considered statistically significant.

Results and discussion

Paper I

Effects of indigestible carbohydrates in barley on glucose metabolism, appetite and voluntary food intake over 16 h in healthy adults

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The purpose of Paper I was to evaluate the effects of intrinsic indigestible carbohydrates in boiled barley kernels (BK) ingested as a late evening meal, on metabolic test markers at fasting and in the postprandial phases following breakfast and lunch the next day. A white wheat bread (WWB) was included as a reference product. The breakfast and lunch were standardized with respect to quality, but consumed ad libitum. The test variables included were markers of glucose regulation, perceived appetite sensations, appetite regulatory hormones, markers of gut fermentative activity, inflammatory markers, and voluntary food intake. Nineteen healthy, young adults with normal BMI, were enrolled in the study. The evening test and reference meals were consumed in random order using a cross-over design.

Results

The results revealed a significant improvement in postprandial glycaemic response during the experimental day after ingestion of BK in the evening compared to the reference WWB. A decrease in b-glucose iAUC by 34% after the BK was shown when taking both the breakfast and lunch meal into consideration (0-330 min, $P < 0.01$). The improvement was, however most pronounced after the breakfast with significant decreases in both b-glucose iPeak and iAUC 0-120 min (-33%, $P < 0.001$ and -41%, $P < 0.001$,

respectively). In addition, the p-insulin iPeak was decreased after breakfast following the BK evening meal (-16%, $P < 0.05$). The BK evening meal generated higher GP[2] than the reference WWB, manifested by a lowered iPeak and a low but sustained net increment in postprandial glycaemia. This was yet another indication of an improved postprandial course of glycaemia.

In addition to improved glycaemic regulation, the BK evening meal increased gastrointestinal hormones of importance for glucose regulation and appetite control. At fasting and during the entire experimental day, elevated concentrations of circulating p-GLP-1 were observed after the evening meal with BK as compared to the evening WWB meal (fasting, +20%, $P < 0.05$ and AUC 0-330 min, +34%, $P < 0.01$, respectively). Furthermore, after the BK evening meal there was a strong tendency towards a reduction in fasting concentrations of p-ghrelin (-16%, $P = 0.07$). In comparison to the WWB, the BK evening meal resulted in decreased subjective ratings of hunger during the whole experimental day (AUC 0-330, -12%, $P < 0.05$) and it was strongest prior to serving of the lunch (AUC 120-210 min, -14%, $P < 0.05$). At lunch, the voluntary energy intake was reduced by 12% after BK evening meal compared to the reference WWB meal ($P < 0.05$).

The BK evening meal resulted in a less pronounced decrease in s-adiponectin concentrations at the end of the experimental day compared with the WWB evening meal (-1.4% and -7.9% for BK and WWB, respectively, $P < 0.05$). In addition, the fasting concentration of circulating s-FFA was significantly decreased (-18%, $P < 0.05$) after the BK evening meal. Further, a tendency towards decreased fasting s-IL-6 concentrations was observed (-18%, $P = 0.06$), when compared to the evening meal with WWB.

The BK evening meal resulted in a significant increase in breath H_2 in the morning compared to WWB, indicative of increased gut fermentative activity.

Discussion

The present paper shows that a BK evening meal had the potential to improve glycaemic regulation over the course of the experimental day. Moreover, the BK evening meal significantly reduced voluntary energy intake at lunch compared to the reference evening meal with WWB. Interestingly, concentrations of p-GLP-1 were increased over the course of the entire experimental day following the BK evening meal. GLP-1 has been attributed

properties important in both glucose- and appetite regulation. It has been observed that intravenously infused GLP-1 in the morning to healthy, normal-weight subjects enhanced satiety and fullness and reduced spontaneous energy intake (-12%) at a subsequent ad libitum lunch meal[146]. The result of the present paper thus presents an interesting approach to increase endogenous GLP-1 concentrations by consumption of BK with accompanying benefits on appetite regulation. Such an approach is further substantiated by the observed association between p-GLP-1 levels and decreased feeling of hunger ($r = -0.72$, $P < 0.01$) as well as to decreased desire to eat ($r = -0.60$, $P < 0.05$), in the morning after BK evening meal (unpublished data).

Increased breath H_2 were observed during the entire experimental day following the evening meal with BK, compared to WWB evening meal. The results indicates possible modulations of gut microbiota, promoting an increased gut fermentative activity. Circulating SCFA, derived from gut fermentation of indigestible carbohydrates have been ascribed signaling properties, with receptors in various peripheral tissues, such as adipose tissue, inflammatory cells, pancreatic islets and within the intestinal tract, altogether consistent with a role in e.g. obesity, T2D and inflammatory diseases[147, 148]. Additional analysis of s-SCFA were performed at fasting and at time 60 and 120 min after the breakfast, respectively, in order to complement the data of the published paper. The results show a main effect of evening meal (0-120 min, $P < 0.05$) revealing higher concentrations of s-propionate at breakfast on the experimental day (fasting, +13%, $P < 0.05$; mean 0-120 min, +15%, $P < 0.05$; unpublished data). There was also a tendency to increased s-butyrate after BK evening meal compared to WWB (+8-9%). The s-acetate concentration corresponded to over 90% of total s-SCFA and if instead combining only the concentrations of s-propionate and s-butyrate a significant increase was observed in these SCFA after BK evening meal at fasting (+11%, $P < 0.05$) and during 2 h following breakfast (+12%, $P < 0.05$), compared to reference WWB evening meal.

Further, a positive relation between p-GLP-1 and p-propionate ($r = 0.64$, $P < 0.05$) was seen at fasting after the BK evening meal. Cani *et al.* (2007) observed that prebiotic (oligofructose) feeding for 4 weeks increased p-GLP-1 levels by promoting an increase in number of L-cells in rats[149]. The mechanism was suggested to be mediated by SCFA-related mechanisms. Involvement of SCFA in regulating GLP-1 release is further supported by *in vitro* studies showing a direct link between SCFA activation of L-cells and increased GLP-1 secretion[94, 150]. In accordance, reduced circulating basal and glucose-stimulated GLP-1 concentrations were found in knockout mice without functioning

SCFA-receptors when compared to wild-type controls[94]. The role of SCFA in glucose- and appetite control has been examined following dietary supplementation with acetate, propionate and butyrate, respectively, during 4 weeks, in a diet-induced obese mice model[151]. The authors demonstrated that SCFA supplementation significantly blocked high-fat diet induced weight gain, and improved oral glucose tolerance and increased circulating concentrations of GIP, GLP-1 and PYY. Increased concentrations of circulating SCFA by virtue of being a marker of gut fermentation thus provide a link between gut fermentation of indigestible carbohydrates and benefits on glucose metabolism and appetite regulation. Accordingly, it could be put forward that the benefits on glycaemia and appetite regulation observed during the experimental day following the BK evening meal is likely to emanate from stimulation of gut fermentative activity, promoted by the specific mixture of indigestible carbohydrates present in this meal. In contrast, it was previously observed by Nilsson *et al.* (2008) that flour-based test meals (barley NSP-enriched white wheat flour bread and WG barley flour porridge, respectively), designed to provide similar amounts of non-starch barley DF as a BK evening test meal, did not induce benefits on over-night glycaemia compared to a reference WWB evening meal[63]. This suggests that the NSP *per se* as present in WG barley product does not affect glycaemic regulation within this time frame. Thus additional characteristics of the intact kernel are likely to contribute to the over-night benefits of barley kernel based evening products. Nilsson *et al.* (2008) further reported that milling of the intact barley kernels in order to produce WG barley flour decreased the RS-content from 7.3 g/meal in the intact kernels to 0.7 g/meal in the WG barley flour porridge[63]. To further explore the metabolic effects of the different components in the barley kernel, WWB supplemented with either high amylose maize RS, or a combination of RS and NSP to mimic the DF content of the whole barley kernel were given to test subjects in an over-night study. At a sub-sequent standardized breakfast meal, improvements of glucose tolerance were seen after the combined RS + NSP test meal, but not after RS supplementation of the test meal only, compared to a reference WWB[64].

It has been stated that the intact botanical structure of cereals is important for inducing benefits on glucose and insulin responses acutely after consumption[53]. In a semi-acute over-night perspective, as is described in the present paper, the botanical structure of the cereal products appears to be essential[63, 64, 152]. Hence, the intact botanical structure together with the intrinsic DF, altogether seems to augment the over-night effects of

intact barley kernels compared to WG barley flour based products or a barley NSP- and RS-enriched WWB[63, 64]. The benefits on metabolic regulation of an intact cereal structure may be due to the accompanying low-GI features. However, when comparing over-night effects of low-GI pasta vs. high-GI WWB evening meals, no benefits were seen within this time frame with the low-GI pasta[153]. It was concluded recently in a review of 16 acute meal studies that the intact barley kernel and the presence of specific DF, such as β-glucans, is crucial to significantly reduce postprandial glucose response[154]. The results presented in the present paper thus contributes with knowledge of the metabolic benefits of barley kernel based products in an over-night perspective. Increased gut fermentation is suggested to be of crucial importance in the over-night perspective, as oppose to the low-GI features in the acute perspective referred to above.

The present paper observed a reduced decline in p-adiponectin over the course of the day after the BK evening meal compared to the WWB. Inconclusive data are present regarding the postprandial response of adiponectin among healthy and metabolically disturbed subjects[155-158]. However, increased concentrations of adiponection have been associated with decreased body weight and improved insulin sensitivity and inflammation[159-161]. When assessing qualitative food aspects in healthy subjects the maintenance of adiponectin level could be considered as positive in relation to metabolic disorders. Further, associations between low-GI diets, high cereal fiber intake and increased plasma adiponectin concentrations have been observed among T2D men[162]. Also among apparently healthy women, high consumption of WG cereals were positively associated with adiponectin concentrations supportive of a potentially beneficial role of dietary factors that promote higher adiponectin levels.

Previous "over-night" studies investigating food related effects on cardiometabolic risk markers have included a "standardized" breakfast with respect not only to nutrient composition, but also to meal size. In contrast, the presently described study included an ad libitum design at both the breakfast and lunch meals thus providing a more realistic eating situation. At breakfast there were no differences in voluntary energy intake depending on previous evening meal (BK evening meal -4%, $P = 0.6$). Thus, it is highly unlikely that the differences observed in glycemic- and metabolic responses to the breakfast meal could be explained merely by the differences in energy intake at this meal.

Taken together, improved glucose tolerance, increased p-GLP-1 concentrations, decreased inflammatory markers, s-FFA and hunger sensations, and reduced energy intake at a

subsequent lunch were observed after an evening meal with BK when compared to reference WWB. Indicative of an amplified colonic fermentative activity, increased concentrations of breath H_2 and circulating s-SCFA were observed 10.5 h after BK consumption. The observed benefits on glycaemia and appetite regulation are suggested to emanate from increased gut fermentation of the indigestible carbohydrates present in the BK meal. The suggested mechanisms may contribute to the metabolic benefits of WG diets reported on T2D[18, 19], weight maintenance[21-23] and CVD[20] in observational studies.

Paper II

Increased concentrations of gut hormones and insulin sensitivity index following three days intervention with a cereal product rich in indigestible carbohydrates in healthy middle aged subjects; a randomized cross-over study

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Submitted Manuscript

The purpose was to investigate the role of indigestible carbohydrates present in a barley kernel based bread (BB) on metabolism and gut hormones at a subsequent standardized breakfast. The study was performed in a cohort of healthy, middle-aged subjects (50-70 years) as opposed to young subjects (20-35 years) studied previously (Paper I). The study had a randomized cross-over design and involved 20 healthy, normal to slightly overweight, middle-aged subjects. The intervention time with BB or white wheat bread reference (WWB) was prolonged to three consecutive days prior to the over-night study rather than providing the test- or reference products as a single evening meal (Paper I). The range of blood parameters was extended to include measures of glucose metabolism, gut derived hormones and inflammatory markers. Gut fermentative activity was determined and subjective appetite sensations were registered.

Results

Following priming for 3 days, a BB test product facilitated glucose regulation at a subsequent standardized breakfast meal compared with corresponding intervention with a reference product, WWB. In the morning at day four after BB, both iPeak and iAUC 0-120 min were significantly decreased for b-glucose (-17%, $P < 0.05$; -22%, $P < 0.05$) and s-insulin (-11%, $P < 0.05$; -17%, $P < 0.01$). In addition, $ISI_{composite}$ was significantly increased at the standardized breakfast after BB intervention period, compared to WWB (+25%, $P < 0.01$), indicative of improved insulin sensitivity following BB.

Increased gut fermentative activity as indicated by elevated breath H_2 concentrations was observed at fasting (+146%, $P < 0.01$) and remained higher during 2.5 h of measurements (mean 0-150 min, +363%, $P < 0.001$) after three days intervention with

BB as opposed to WWB. Higher total s-SCFA levels (acetate, butyrate and propionate; +18%, P <0.05) were observed at fasting after intervention with BB compared to WWB. More specifically, s-acetate concentration was significantly increased by 18% (P < 0.05). S-butyrate (+13%, P = 0.1) and the sum of s-propionate and s-butyrate tended to increase, although not statistically significant (+11%, P = 0.07, data not shown) after BB intervention compared to WWB.

Further, increased levels of circulating gut hormones were observed after BB intervention at fasting in the case of p-GLP-1 (+56%, P < 0.01) and during the experimental day for p-GLP-2 and p-PYY (mean 0-150 min, +13% and +18%, P < 0.05, respectively). Moreover, a main effect of test meal was observed on p-PYY concentrations (P < 0.05) revealing higher levels following BB compared to WWB.

Total s-SCFA at fasting were significantly and positively associated with p-PYY both at fasting (r = 0.51, P < 0.05 and r = 0.57, P < 0.01, after WWB and BB, respectively) and during the standardized breakfast (mean 0-150 min, r = 0.51, P < 0.05 and r = 0.55, P < 0.05, after WWB and BB, respectively). In addition, fasting total s-SCFA was positively associated with p-GLP-2 after the BB intervention (mean 0-150 min, r = 0.53, P < 0.05) and tended to be related after WWB intervention (mean 0-150 min, r = 0.40, P = 0.09).

No significant differences were observed in p-OXM, p-ghrelin, s-FFA, s-IL-6, s-IL-18 or s-adiponectin depending on intervention product. Subjective appetite sensations did not differ depending on intervention product. However a tendency for a treatment effect appeared for desire to eat (P = 0.08) with less desire to eat after a three day intervention period with BB.

Discussion

The present paper shows that intake of BB during three days improved glycaemic regulation and measures of insulin sensitivity in the perspective from an evening meal to a subsequent standardized breakfast, as opposed to a corresponding intervention period with WWB. This outcome of BB ingestion is of potential importance in that the cohort studied can be considered at risk to develop T2D due to their age. Accordingly, deterioration in glucose tolerance is associated with age[163, 164] and the prevalence of T2D was reported to increase with age in different populations[165, 166]. Interestingly, in the present work increased insulin sensitivity index ($ISI_{composite}$), indicative of improved insulin

action, was observed among test subjects. This is in contrast to previous work including a single evening test meal with barley kernel based product[74, 157] and results in this paper indicates not only improvement in glucose responses at breakfast but also significantly reduced s-insulin iPeak and iAUC 0-120 min after the standardized breakfast. The more profound effects on glucose metabolism could be due to the extended intervention period with priming for three days prior to the over-night study. Other contributing factors could be the anticipated, age-related decline in glucose tolerance, as discussed above, making the cohort more sensitive to dietary modulations of insulin sensitivity.

GLP-1, PYY and GLP-2 are co-stored in secretory granules within the endothelial L-cell, and are suggested to be co-released upon stimulation[167]. Previously, co-release of PYY and GLP-1 from L-cells was reported based on studies in human and/or mice colonic mucosa (*in vitro*)[168, 169]. Whereas GLP-1 and PYY act as signaling molecules targeting both neuronal regions and specific tissues, e.g. pancreas, GLP-2 possesses important local features in the intestinal epithelium by e.g. enhancing cell proliferation and increasing mucosal mass, altogether expanding the gastrointestinal mucosal surface area[107]. It was observed by Thulesen *et al.* (1999) that a DF-rich diet during 3 weeks significantly increased intestinal weight, compared to a DF-free diet in diabetic rats, primarily by growth of the mucosal layer, and plasma levels of GLP-2 paralleled the intestinal growth[170]. Cani *et al.* (2009) reported that prebiotics (oligofructose) lowered systemic inflammation by decreased circulating LPS and cytokine levels, and decreased intestinal permeability in genetically obese mice[108]. The authors proposed that the improved gut barrier functions observed after prebiotic feeding, were controlled by selective gut microbial changes and increased GLP-2 concentration[108]. Further, pharmacological treatment with GLP-2 induced changes similar to prebiotic-feeding, whereas GLP-2 antagonist abolished most of the effects, supportive of a GLP-2-dependent mechanism related to gut microbiota changes and gut barrier functions; with potential benefit on systemic inflammation[108]. The role of intestinal integrity emerge as an important factor in disorders related to the MetS. The results presented in this paper are among the first to show increased p-GLP-2 levels in response to gut fermentation of DF-rich foods in healthy humans as measured with the over-night experimental design used. Similarly, a 5-week intervention period with inulin-enriched pasta as opposed to control pasta, increased fasting p-GLP-2 and decreased markers of intestinal permeability in healthy young men[171]. The physiological relevance of increased levels of circulating gut hormones involves mechanisms important to glucose- and appetite regulation, but also for intestinal

barrier function, and inflammatory tonus. However, no effects on circulating inflammatory markers (s-IL-6 and s-IL-18) were observed depending on intervention period with BB or WWB, respectively in the present work. This result was unexpected, because of the previously observed effects on primarily circulating IL-6 in in response to barley-based products[64, 172, 173].

The possibility to stimulate the release of endogenous hormones, as a true physiological mixture of gut peptides, has been suggested as future targets for drug development as opposed to the current advance in pharmacological treatments with e.g. GLP-1 based therapies in T2D[167]. Egerod *et al.* (2012) described that co-expression and co-storage of several gut hormones, including GLP-1 and PYY, were found in enteroendocrine cells throughout the human small intestine (*in vitro*) and suggested that the expression patterns potentially could be modulated by environmental factors[174]. The present paper describes such a possibility for co-release of gut peptides by the consumption of a specific combination of indigestible carbohydrates present in BB. Hence, in addition to increased p-GLP-2, three days intervention with BB increased also concentrations of p-GLP-1 and p-PYY compared to reference WWB, indicative of a simultaneous increase of co-released gut hormones in response to intrinsic DF in a composite food. The results indicate that specific dietary regimes including e.g. barley kernel based products could be considered beneficial in relation to glucose- and appetite regulation, but also with respect to improved intestinal integrity, among elderly healthy subjects.

Recently, it was stated that MetS-associated components, e.g. impaired glucose tolerance and abdominal obesity, may be important in the development of different states of cognitive decline; from an age-related decline in cognitive function to diagnosed neurodegenerative diseases, such as Parkinson's and Alzhemier's disease[175]. The common feature is proposed to be the impaired insulin action within the central nervous system (CNS) present in e.g. T2D and Alzheimer's disease[176]. Moreover, increased age is related to the progress of "normal" cognitive decline[177] but also with higher prevalence of neurodegenerative diseases[178, 179]. Thus in an older population, strategies to prevent neuronal degeneration, involved in age-related deterioration of cognitive function, could be regarded as advantageous in promoting a more "healthy" cognitive ageing. Neuroprotective effects have been observed by GLP-1[180] and it has been reported that plasma concentration of GLP-1, but also GLP-2, within the *in vivo* range, promotes neuronal survival in enteric neurons (*in vitro*)[181]. The neuroprotective effects are suggested to be mediated by direct stimulation of neuronal receptors, involving both GLP-1 and

GLP-2 receptors[181, 182], and have been demonstrated not only in enteric neurons but also in neurons within the CNS[183, 184]. The impaired insulin action within the CNS associated with cognitive decline, implies that incretin-based therapies targeting insulin action may be advantageous also in prevention of neurodegenerative diseases. The potential neuroprotective effect of GLP-1 and GLP-2 is noteworthy in relation to the increase of these gut hormones observed after the BB intervention period in the older study group included in the present paper.

The results in in the present paper indicates that three days intake of BB significantly impacts gut fermentative activity as estimated by increased breath H_2 both at fasting and during the experimental day, but also in relation to increased concentrations of s-SCFA. The proportion of acetate in relation to propionate and butyrate accounts for the majority of the distribution of total SCFA. However, it can be noticed that when adding the s-propionate and s-butyrate, a strong trend towards increased concentrations of these acids emerge following BB intervention period (+11%, $P = 0.07$). In addition, s-butyrate was increased by approximately 13% although not statistically significant but altogether contributing to the significant increase in total s-SCFA concentrations. Receptors for SCFA are present in both colonic- and peripheral tissues which thus promotes a role of SCFA-signaling in e.g. GLP-1 secretion from enteroendocrine L-cells and lipolytic activity in adipocytes, in accordance with a coordinating role in metabolic processes[147, 148]. The results of in the present paper add knowledge to the possibility of inducing metabolic benefits by consumption of barley kernel based products, also among ageing subjects. As described in Paper I the effects are possibly mediated by gut microbial metabolites, such as SCFA, and concomitant release of specific gut hormones. The hypothesis is further strengthened by a correlation observed between total s-SCFA and p-PYY and also between total s-SCFA and p-GLP-2 in the present paper.

Taken together, three days intervention with DF-rich BB significantly increased concentrations of the gut hormones p-GLP-2, p-PYY and p-GLP-2, facilitated blood glucose regulation and increased insulin sensitivity index, increased total s-SCFA and increased breath H_2 indicative of increased colonic fermentative activity in middle-aged subjects. In addition, circulating s-SCFA were positively correlated to p-PYY and p-GLP-2, in support of a link between gut microbial fermentation of indigestible carbohydrates and benefits on host metabolism. Thus metabolic benefits of indigestible carbohydrates in young healthy adults previously presented in Paper I, appears to be

present also among an ageing study population, providing a tool in prevention of age-related metabolic disorders and possibly cognitive decline.

Paper III

Effects of a Brown Beans Evening Meal on Metabolic Risk Markers and Appetite Regulating Hormones at a Subsequent Standardized Breakfast: A Randomized Cross-Over Study

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The purpose of Paper III was to evaluate the effects of intrinsic indigestible carbohydrates in boiled legumes ingested as a late evening meal, on metabolic markers at fasting and in the postprandial phase following a subsequent standardized breakfast. It was hypothesized that the indigestible carbohydrate components present in legumes could support gut fermentation and mediate benefits on glucose- and appetite regulation as previously reported for barley kernel products rich in intrinsic indigestible carbohydrates.

Included as a late evening meal were boiled beans (*Phaseolus vulgaris* L.) of different varieties (red "kidney", white "Cannellini", brown and black) and chickpeas (*Cicer arietinum*). A WWB was included as an evening reference meal. The boiled legumes were investigated with respect to glycaemia, insulinaemia, perceived appetite sensations, and markers of gut fermentative activity (breath H_2). Plasma was selected from subjects following the brown bean (BrB) evening test meal and from the WWB reference evening meal for extended analysis with respect to gut hormones (GLP-1, GLP-2, OXM and PYY), ghrelin, SCFA and inflammatory variables (IL-6 and IL-18).

Results

The composition of starch (total and available) and DF components of the different beans included in the present paper is presented in Table 6 (page 64)

Significant main effects of treatment were observed for both b-glucose and s-insulin responses (0-120 min, $P < 0.05$) revealing decreased response to the standardized breakfast meal after BrB evening meal compared to WWB evening meal. BrB significantly decreased both iPeak (-15%, $P < 0.05$) with respect to b-glucose, as well as decreased iAUC (0-120 min) for both b-glucose (-23%, $P < 0.05$) and s-insulin (-16%, $P < 0.05$) at

the subsequent breakfast, compared to WWB. No differences were observed at fasting for b-glucose or s-insulin, respectively, depending on the previous evening meal.

The fasting breath H_2 values after evening meals with chickpeas, white beans or BrB were significantly higher compared with the reference WWB (+59%, $P < 0.01$; +118%, $P < 0.001$ and +100%, $P < 0.001$, respectively). In addition, during postprandial measurement 11-14 h after intake of the evening test meals, all five legumes induced higher concentrations of breath H_2 compared with the reference WWB (mean 0-180 min, +90-153%, $P < 0.05$-0.001).

The subjective feeling of satiety was significantly increased at fasting after intake of boiled chickpeas as a late evening meal, compared to WWB (+104%, $P < 0.05$).

The BrB evening meal showed the most beneficial over-night effects on glucose tolerance in combination with high breath H_2 and was therefore chosen for further analyses of gut hormones, SCFA and inflammatory markers. The physiological variables and perceived appetite sensations 11-14 h after evening meals with different legumes are presented in Table 7 (page 65).

Significant main effects of treatment were observed for appetite regulatory hormones after the standardized breakfast (0-180 min, p-PYY, $P < 0.0001$; p-OXM, and p-ghrelin, $P < 0.05$). Further, at fasting after BrB evening meal, concentrations of p-PYY were significantly increased (+53%, $P < 0.01$) and remained elevated during the experimental period after the standardized breakfast (mean 0-180 min, +51%, $P < 0.05$) compared to reference WWB. No differences in p-GLP-1 concentrations at fasting nor following the standardized breakfast were observed depending on evening meal. A tendency towards a significant meal × time interaction was observed for p-GLP-2 concentrations in the postprandial period after the breakfast ($P = 0.07$) making it relevant to investigate different time points and intervals. Thus, the BrB evening meal significantly increased p-GLP-2 concentrations in the late postprandial period after the standardized breakfast (AUC 60-180 min, +8%, $P < 0.05$) compared to reference WWB. The orexigenic hormone ghrelin was significantly decreased (AUC 0-180 min, -14%, $P < 0.05$) after the standardized breakfast post BrB evening meal compared to WWB. Further, inverse relations between the willingness to eat (0-45 min) and p-PYY (mean 0-180 min, $r = -0.50$, $P < 0.05$) and p-GLP-2 (mean 0-180 min, $r = -0.51$, $P < 0.05$), respectively, were observed during the breakfast after BrB evening meal.

The perceived sensation of hunger was decreased in the early time period after the standardized breakfast (AUC 0-45 min, -15%, $P < 0.05$) post the BrB in comparison with WWB evening meal. Moreover, following BrB, p-OXM concentrations (mean 0-180 min) tended to correlate to the subjective feeling of satiety (AUC 0-45 min) ($r = 0.48$, $P = 0.06$).

Both p-propionate and p-isobutyrate levels were significantly increased (mean 0-60 min, $P < 0.05$ and $P < 0.001$, respectively) on the subsequent morning after BrB compared with after the WWB.

Significant main effects of evening meals were observed on inflammatory markers s-IL-6 and s-IL-18 at the standardized breakfast (0-180 min, $P < 0.05$). Mean concentrations throughout the postprandial phase after breakfast (0-180 min) were decreased for both s-IL-6 and s-IL-18 (-35%, $P < 0.05$ and -8%, $P = 0.05$, respectively). A significant time × treatment interaction ($P < 0.001$) was observed concerning s-IL-18, revealing decreased concentrations at 120 min after the standardized breakfast (-28%, $P < 0.001$) the morning after BrB, compared to after WWB.

Table 6. Composition of starch (total and available) and DF components in the legume evening products and reference WWB, respectively[1]

	Starch			NSP			Total DF
Products	Total	Available	RS	Insoluble	Soluble	Raffinose[2]	
	%dry matter						
Chickpeas	50.7	44.2	6.5	15.9	3.3	4.3	30.0
White beans	43.1	37.5	5.5	17.7	8.1	3.1	34.5
Red beans	42.2	33.5	8.7	19.7	7.2	2.2	37.7
Black beans	45.8	39.9	6.0	20.4	3.4	1.3	31.1
Brown beans	48.9	41.3	7.6	15.4	9.4	3.2	35.7
WWB	79.7	77.3	2.5	3.8	0.6	0.1	6.9

[1]Values of available starch are based on means of 2 replicates, RS means of 6 replicates, insoluble and soluble NSP means of 3 replicates, raffinose means of 2 replicates. Available starch was calculated by subtracting RS from total starch. Included in total DF are RS analyzed as described by Åkerberg *et al.* (1998)[138], insoluble- and soluble NSP determined gravimetrically according to Asp *et al.* (1983)[139] and raffinose as described in section: Chemical analysis of the test- and reference products and meals. DF, dietary fibre; NSP, non-starch polysaccharide; RS, resistant starch; WWB, white wheat bread (reference).

[2]The α-galactosidase in the kit used for quantification of raffinose, also hydrolyses other α-galactosides, such as stachyose and verbascose.

Table 7. B-glucose, s-insulin, breath H_2 and feeling of satiety, hunger and desire to eat 11-14 h after intake of five different legume evening test meals or a reference WWB[1]

	WWB	Chickpeas	White beans	Red beans	Black beans	Brown beans
b-glucose, iAUC 0-120 min (*mmol·min/l*)	192 ± 14.5	168 ± 17.9 (-13%)	161 ± 11.7 (-16%)	157 ± 15.7 (-18%)	168 ± 19.9 (-13%)	**147 ± 8.84* (-23%)**
b- glucose, iPeak (*mmol/l*)	3.1 ± 0.2	2.9 ± 0.19 (-11%)	2.8 ± 0.19 (-11%)	2.8 ± 0.19 (-9%)	2.8 ± 0.26 (-11%)	**2.7 ± 0.17* (-15%)**
s-insulin, iAUC 0-120 min	16.6 ± 1.33	15.8 ± 1.67 (-5%)	14.1 ± 1.43 (-15%)	15.4 ± 1.68 (-7%)	15.5 ± 1.49 (-7%)	**13.9 ± 1.45* (-16%)**
Breath H_2, fasting values (*ppm*)	17 ± 2.6	**27 ± 3.4** (59%)**	**37 ± 6.7*** (118%)**	23 ± 4.0 (35%)	25 ± 3.9 (47%)	**34 ± 5.4*** (100%)**
Breath H_2, mean 0-180 min (*ppm*)	7.9 ± 2.8	**17 ± 3.0*** (115%)**	**20 ± 5.6*** (153%)**	**15 ± 4.2* (90%)**	**15 ± 4.1** (90%)**	**19 ± 4.5*** (141%)**
Satiety, fasting value (*mm*)	14.2 ± 3.5	**29.0 ± 7.5* (104%)**	18.5 ± 4.9 (30%)	22.4 ± 7.1 (58%)	16.8 ± 5.8 (18%)	20.4 ± 6.8 (43%)
Satiety, AUC 0-180 min (*m·min*)	6.2 ± 0.8	6.8 ± 1.0 (9%)	6.3 ± 0.8 (2%)	6.2 ± 0.8 (0.4%)	6.9 ± 0.9 (11%)	6.7 ± 0.8 (8%)
Hunger, AUC 0-180 min (*m·min*)	9.8 ± 0.9	9.1 ± 1.0 (-7%)	9.6 ± 0.9 (-2%)	9.7 ± 1.0 (-1%)	9.4 ± 0.9 (-5%)	9.1 ± 1.0 (-7%)
Desire to eat, AUC 0-180 min (*m·min*)	11.0 ± 1.0	10.3 ± 1.1 (-6%)	10.3 ± 0.8 (-6%)	11.3 ± 1.0 (3%)	10.5 ± 1.0 (-5%)	10.0 ± 1.0 (-9%)

[1]Values are presented as means ± SEM. Percentage within brackets indicates change compared to WWB. AUC, area under curve; b, whole blood; H_2, hydrogen gas; iAUC, incremental area under curve; iPeak, incremental peak; s, serum; WWB, white wheat bread.

* Significantly different compared with WWB $P < 0.05$, ** $P < 0.01$ (ANOVA followed by Dunnett's test), n = 16.

Discussion

The purpose of the present study was to investigate if legumes as a source of indigestible carbohydrates were able to induce over-night benefits on glucose- and appetite regulation, as previously described for barley kernel based products. Of importance is to notice the differences in composition of the indigestible carbohydrate fraction between cereals i.e. barley, and legumes. Consequently, in contrast to barley, common beans are rich in indigestible oligosaccharides, mainly raffinose, stachyose and verbascose[185] as well as galactomannans[186]. Other major DF components of beans are cellulose, xyloglucans and arabinose-rich pectins[187]. The indigestible carbohydrates in barley instead consist of an appreciable portion of β-glucans ((1-3),(1-4)-β-D-glucan)[188], but also of cellulose, arabinoxylan and lignin[189]. Alike barley kernel based products, cooked beans are rich sources of RS. Thus the total content of potentially fermentable carbohydrates in cooked beans included in the present study amounted to approximately 33.8 ± 1.4% dry matter versus about 23.3 ± 1.5% dry matter in barley kernel based products (Papers I, II and IV). Taken together, cooked beans, alike kernel based cereal products, consists of appreciable amounts of indigestible carbohydrates that are potentially available for fermentation. A summary of the composition of starch and DF components in BrB versus barley kernels used in the thesis are presented in Table 2 (page 43).

In vitro fermentation studies with polysaccharide extracts from beans by using human fecal microbiota show that indigestible substrates present were readily fermented with production of SCFA (acetate, propionate and butyrate)[185]. Moreover, during fermentation of bean polysaccharides, an extended production of SCFA was reported (*in vitro*) with SCFA concentration increasing in media up to 24 h[185, 190]. Studies in rats confirm the high fermentability of indigestible carbohydrates present in *Phaseolus vulgaris*[191]. The results in the present work indicates that all legumes included increased gut fermentative activity in an over-night perspective as indicated by increased breath H_2 concentration measured at a subsequent reference breakfast of high-GI and low DF content. In the case of the BrB evening meal p-SCFA analyses indicated increased concentrations of p-propionate compared to the WWB evening meal. In addition, also p-isobutyrate increased after BrB. Branched chain fatty acids (BCFA), such as isobutyrate, are formed during microbial degradation of proteins and amino acids (proteolytic fermentation), and are generally associated with mechanisms unfavorable to gut health[192].

For example, studies are available showing increased BCFA in intestinal inflammatory disorders, i.e. ulcerative colitis and Crohn's disease[193]. However, few studies have assessed the role of BCFA gut metabolites in relation to host metabolism and studies investigating fermentation metabolite profiles, most often focus on the SCFA. One reason for the lack of data regarding BCFA formation could be related to that the vast majority of studies included carbohydrate sources low in protein content. However, it could be proposed that a leguminous diet, though providing substantial amounts of indigestible carbohydrates also contributes with considerable amounts of indigestible dietary protein with the potential of providing substrate for proteolytic fermentation. It was observed by Beher-Sehlmeyer *et al.* (2003) that *in vitro* fermentation of different DF-rich sources e.g. inulin, wheat and linseed with human gut microbiota increased SCFA and BCFA, e.g. isobutyrate and isovalerate[194]. Taken together, although not commonly reported, increases in circulating concentrations of BCFA may occur after intake of certain DF-rich foods. However, the metabolic effects of BCFA remain to be established.

The low *acute* glycaemic response of leguminous foods were observed by Jenkins *et al.* already in 1981[50]. Benefits of legumes on glycaemic control have since then also been observed in longer dietary interventions, and a meta-analysis of 41 trials (≥ 7 days) concluded that dietary pulses (chickpeas, beans, peas, lentils etc.) decreased fasting glucose and insulin in both diabetic and non-diabetic subjects[195]. However, the present paper is among the first to report *over-night* effects of legumes on glycaemic response and related metabolic variables in healthy subjects, and relate the effects to mechanisms originating from gut microbial metabolism. Thus, intake of boiled BrB as a late evening meal significantly reduced both b-glucose- and s-insulin responses at a subsequent standardized breakfast, as compared to after a WWB evening meal. Previously benefits on glucose control of barley kernel based products were observed and suggested to originate from events related to colonic fermentation of the indigestible carbohydrates (Papers I and II). The present work suggests that similar mechanisms may be present also after an evening meal with BrB, indicated by increased markers of gut fermentative activity (breath H_2 and p-SCFA). Accordingly, in addition to the glycaemic benefits of a BrB evening meal, important effects were observed also on appetite regulatory hormones p-PYY, p-OXM and p-ghrelin at the subsequent breakfast, 11-14 h after bean intake. In addition, also the gut hormone p-GLP-2 was increased after BrB intake. GLP-2, PYY, and OXM together with GLP-1 are co-released upon stimulation of the gut endothelial L-cell[196, 197]. Enteroendocrine L-cells have been shown to express SCFA receptors in the human large

intestine[92] and rectal infusions with SCFA (acetate), compared to saline or intravenous infusions of SCFA, induced increased levels of plasma PYY in hyperinsulinaemic subjects[198]. The results of presented here thus indicate that the higher levels of p-PYY, p-GLP-2 and p-OXM may be induced by increased production of SCFA emanating from an increased gut fermentation of indigestible carbohydrates as present in the BrB evening meal. PYY, OXM and GLP-1 are potential mediators of the so-called "ileal break", a feedback mechanism that delays intestinal motility in response to unabsorbed nutrients being present in the ileum, hence promoting satiety[196]. Even if the present study did not reveal increased plasma concentrations of GLP-1, the increased levels of p-PYY and p-OXM are consistent with a possible role of BrB in appetite regulation.

As mentioned above, also other hormones involved in appetite regulation was affected and postprandial concentrations of ghrelin (AUC 0-180 min) were reduced in the morning after the BrB evening meal compared with after the WWB. Ghrelin is, in contrast to the other gut hormones discussed, synthesized primarily in the stomach or proximal small intestine and act to promote the sensation of hunger by stimulating the vagus nerve or by acting directly in specific regions within the CNS[199, 200]. The concentrations of circulating ghrelin is thus high in the fasted state and decline rapidly in response to a meal. Intravenously administered ghrelin increased energy intake and subjective ratings of appetite, compared to saline infusions in healthy subjects[201]. A tendency to decreased fasting ghrelin levels (-16%, $P = 0.07$) was observed also in Paper I, following an evening meal with boiled barley kernels. However, in contrast to the results after BrB, postprandial measurements during the experimental day did not reveal any differences in p-ghrelin depending on preceding evening meal (Paper I), The role of different macronutrients in inducing ghrelin secretion is not clear. However it has been observed that infusion of glucose and amino acids to intestinally cannulated (gastric, duodenal and jejunal) rats suppressed ghrelin more rapidly and stronger than lipids[202]. Further, Foster-Schubert *et al.* (2008) observed that the magnitude of acute p-ghrelin suppression in healthy subjects after intake of beverages composed of the macronutrients was in the following order: protein > carbohydrates > fat, respectively[203]. Other studies observed decreased ghrelin levels in healthy subjects in the acute response to carbohydrates, but not to protein and fat[204]. In the present paper it can be suggested that the lowering effects on ghrelin observed in the semi-acute perspective (11-14 h) after the BrB probably are mediated by a mechanism involving the indigestible carbohydrates, and possibly to some extent the indigestible protein. Cani *et al.* (2004) reported decreased

fasting levels of ghrelin (8 h after food deprivation) in rats post 3 weeks treatment with fructans compared to non-fructan fed controls[205]. The authors suggested that increased GLP-1 levels in response to prebiotic feeding possibly inhibited the release of ghrelin, as observed after GLP-1 stimulation of isolated rat stomach (*in vitro*)[206]. In addition, also other gut related hormones, such as PYY and OXM, have been shown to suppress ghrelin secretion in human subjects[207, 208]. Mechanisms involving blockage of ghrelin-activated neurons of the CNS by PYY have been suggested, introducing a possible indirect action of certain gut peptides, e.g. PYY and OXM in appetite control[209].

A recent comprehensive investigation regarding the molecular basis for regulation of ghrelin secretion, reported that the SCFA-receptor FFAR2 was expressed and enriched in isolated gastric ghrelin-secreting cell from mice[210] and propionate and acetate were suggested to inhibit ghrelin secretion efficiently through this FFAR2 receptor[210]. Interestingly, among the SCFA, propionate showed strongest potency to bind to FFAR2[147]. In the present work, the evening meal with BrB increased circulating levels of p-propionate at the subsequent breakfast (mean 0-60 min), while at the same time decreasing p-ghrelin, when compared to WWB evening meal. Similarly, Tarini *et al.* (2010) reported that inulin intake increased levels of circulating propionate and butyrate, and decreased s-ghrelin after 4-6 h in healthy subjects[211]. It can thus be hypothesized that increased levels of circulating SCFA, may act to inhibit the release and action of p-ghrelin, with possible influence on appetite control. Accordingly, in the present paper, the perceived sensation of hunger were decreased in the early time perspective after the standardized breakfast (AUC 0-45 min) after BrB evening meal, compared to WWB.

The present paper reports significant decreases in inflammatory markers (s-IL-6 and s-IL-18) at the standardized breakfast after BrB evening meal in healthy subjects, compared to WWB. In addition to indigestible carbohydrate substrates, legumes as well as cereals, also contain DF-associated bioactive compounds such as polyphenols[212, 213], generally recognized for their anti-inflammatory and antioxidative properties[214]. Moreover, polyphenols may interact with the gut microbiota e.g. as substrate for colonic fermentation and accordingly participates in modulation of the gut eco-system with potential health effects[215]. The beneficial effects on e.g. inflammatory tonus in the present study may thus be influenced to some extent by the polyphenol content in the BrB evening meal. However, the observed increase in p-GLP-2 in response to the BrB evening meal might reflect an improved intestinal barrier function, thus inhibiting influx of pro-inflammatory gut metabolites such as e.g. LPS. Such an effect of GLP-2 was previously

suggested by Cani *et al.* (2009). Hence, intake of indigestible carbohydrates was proposed to induce selective shifts in gut microbiota with concomitant release of GLP-2 and improved intestinal integrity, counteracting development of metabolic endoxemia[108]. In Paper II, three days intervention with barley kernel based bread induced increased gut fermentative activity and increased levels of p-GLP-2 at the subsequent breakfast also in an older study cohort (50-70 years). However, no effects were observed with respect to inflammatory response. The origin and/or content of the indigestible carbohydrate substrates may be of importance for the differences in inflammatory responses seen in Paper II and in the present paper. However, the evening meals with barley kernel products tended to reduce fasting s-IL-6 levels in Paper I (P = 0.06). Similar benefits of a barley kernel evening meal on s-IL-6 were reported by Nilsson *et al.* (2008)[64]. It has further been proposed that any compound influencing the gut microbiota in a way to increase production of SCFA might be expected to influence inflammatory responses, through a mechanism involving the SCFA receptor FFAR2 of the immune cells[216].

In conclusion, an evening meal with BrB, rich in indigestible carbohydrates was capable of improving glucose- and appetite regulation as well as improving inflammatory tonus in healthy subjects at a subsequent standardized high-GI breakfast, 11-14 h after intake. The over-night benefits are probably mediated by mechanisms emanating from gut fermentation and suggest that the specific indigestible carbohydrates intrinsic to boiled legumes may have potential prebiotic effects. The results provides additional evidence for a link between gut microbial metabolism and key factors associated with MetS-related disorders.

Paper IV

Over-night metabolic effects of barley kernel products with or without supplementation with some commercially available probiotics

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Manuscript

The purpose was to investigate if metabolic effects previously observed in healthy subjects with barley kernel based products rich in indigestible carbohydrates, are affected by parallel intake of some common commercially available probiotics. Three different intervention periods were included. A barley kernel based bread (BB) was included during 4 days as part of the normal diet, with or without probiotic "priming" 10 days prior to, and during the actual 4 days intervention with BB (BB(+) and BB(-), respectively). A 4 day intervention period with white wheat bread (WWB) without probiotic supplementation (WWB(-)) was included as reference. The daily bread portions of BB or WWB were divided equally between breakfast- and late evening meals during the 4 days intervention period. At a subsequent standardized WWB breakfast on day 15, blood samples were collected for analyses of markers of glucose regulation, gut hormones (including satiety hormones), inflammatory markers, triglycerides and markers of endothelial function. In addition, subjective appetite sensations and breath H_2 were registered, and voluntary energy intake measured at a following lunch meal. The study was performed as a semi-randomized cross-over study, with the intervention period including probiotic supplementation (BB(+)) last in order. Twenty-one healthy, normal-weight young adults were included in the study.

Results

The b-glucose responses were decreased at the standardized breakfast following the intervention periods including BB compared to WWB(-). However, only the decrease after the BB(-) reached statistical significance compared to WWB(-) (iAUC 0-210 min, -28%, $P < 0.05$ and iPeak, -22%, $P < 0.01$). No differences were observed in b-glucose at the standardized breakfast when comparing BB(+) with BB(-) (iAUC 0-210 min, P =

0.77 and iPeak, P = 0.56, respectively). No significant differences were observed depending on preceding intervention with respect to b-glucose or s-insulin at fasting nor with respect to postprandial s-insulin responses or insulin sensitivity index ($ISI_{composite}$).

Indicative of increased gut fermentative activity, breath H_2 was significantly increased after both intervention periods including BB, compared to WWB(-) (+71-99%, P < 0.05). However no differences were observed between BB intervention periods.

A significant main effect of intervention period were observed for p-PYY (0-210 min, P < 0.01) and a tendency towards effect was observed regarding p-GLP-1 (0-120 min, P = 0.07). Further, p-GLP-1 concentrations were increased during the experimental day following BB(-) and BB(+), respectively, compared to the WWB(-) (mean 0-120 min, +17%, P < 0.01 and +14%, P < 0.05, respectively). Supplementation of the BB with probiotics did not affect p-GLP-1 when compared to non-supplemented BB intervention period (P = 0.54). However, probiotic supplementation of the BB increased concentrations of p-GLP-2 during the experimental day, (+7%, P < 0.05), and p-glucagon levels at fasting (+6%, P < 0.05), compared to non-probiotic supplementation of BB, but not compared with WWB(-).

No differences were observed depending on preceding intervention period in circulating inflammatory markers (s-IL-6, s-IL-18 or s-CRP) at the standardized breakfast at fasting or during the experimental day. After intervention period BB(+), a significant increase in s-PAI-1 levels was observed at fasting, compared to WWB(-), and also when compared to BB(-) (+10%, P < 0.05). s-TG were not significantly different at fasting nor postprandially at the standardized breakfast depending on intervention periods.

Further, intervention periods BB(-) or WWB(-) did not result in differences in perceived satiety, hunger or desire to eat at fasting nor during the experimental day. Neither were there differences in voluntary energy intake at lunch depending on preceding intervention. BB(+) was not included in the analysis of voluntary food intake or perceived sensations of appetite due to lack of randomization in the case of this intervention period.

Discussion

In the presently described study probiotics commonly available on the Swedish market were selected and included in one of the BB interventions as a mixture (*L. plantarum* 299v, *L. reuteri* DSM 17938 and *B. animalis* DN-173 010). The rational for this design

was to investigate whether the gut fermentation mechanism suggested to be the cause of the over-night benefits of barley kernel products on glucose tolerance, inflammatory tonus and satiety[64] could be modulated in a dietary background of these probiotics. Information is at hand suggesting that probiotic supplementation (*B. adolescentis*) induced benefits on insulin sensitivity after 12 weeks in high-fat fed obese rats[217]. Also a probiotic mixture (*L. acidophilus* NCDC14 and *L. casei* NCDC19) was reported to suppress the onset of diabetes in pharmacologically induced diabetic rats after 28 days of treatment[218]. Among diabetic and non-diabetic subjects, probiotic supplementation (*L. acidophilus* NCFM) during 4 weeks preserved insulin sensitivity in contrast to decreased sensitivity in the placebo group[219]. Thus, potential improvements in glucose homeostasis after probiotic supplementation has been observed in both animal and human studies.

No data are available regarding the potential glucose lowering effects of the commercial probiotics selected to be included in the present paper. However, there are reports of increased gut transit time (*B. animalis* DN-173 010)[220, 221], modulation of gut immune system (*L. reuteri* DSM 17938)[222] and lowering of LDL-cholesterol, and increased feacal SCFA (*L. plantarum* 299v)[223, 224].

Studies investigating potential synbiotic effects of pre- and probiotics on metabolic responses in humans are scarce. However, Furrie *et al.* (2005) reported that synbiotics (*B. longum* and oligofructan enriched inulin) consumed during 4 weeks by ulcerative colitis patients (colonic inflammatory disease) reduced expression of inflammatory cytokines (IL-1α and TNF-α in mucosal tissue and increased bifidobacteria by 42-fold compared to baseline[225]. Bomhof *et al.* (2013) observed benefits on glycaemia in diet-induced obese rats after 8 weeks treatment with prebiotics (oligofructose) and/or probiotics (*B. animalis* subsp. *lactis* BB-12) as estimated in an oral glucose tolerance test, compared to non-treated control[226]. The authors further observed increased concentrations of gut hormones PYY and GLP-1 in plasma, and increased abundance of the caecal bacterial genera *Lactobacillus spp.* and *Bifidobacterium spp.* after prebiotic treatment, but not after probiotic or synbiotic treatment[226].

The present paper report that supplementation with commonly available probiotics to barley kernel based products does not appear to induce major modulations of the metabolic outcome with barley kernel based products, as reported in Papers I and II, and previously, in a semi-acute over-night perspective[63, 64, 153].

Increased levels of p-GLP-1 concentrations were observed during the experimental day after 4 days intervention periods including BB, irrespective of probiotic supplementation, compared to WWB(-). Both intervention periods with BB increased concentrations of breath H_2 indicative of a higher state of gut fermentation at the subsequent breakfast, compared to WWB(-) intervention period. No differences between intervention periods with BB(-) and BB(+) were observed with respect to p-GLP-1 and breath H_2 levels, suggesting of that the indigestible carbohydrates present in the BB induced important effects on gut fermentation with a concomitant release of p-GLP-1 regardless of probiotic supplementation. Further, a main effect of intervention period was observed for p-PYY during the experimental day. Though no statistical differences were observed in regards of mean values in the time period 0-210 min after the breakfast for p-PYY (ANOVA) depending on intervention period. However, if instead investigating the mean for p-PYY in the time interval 60-210 min, a strong tendency for increased p-PYY concentrations was observed after the BB(-) compared to the WWB(-) (+6%, $P = 0.07$, data not shown). The previously observed parallel increase of gut hormones as reported in Paper II (p-GLP-1, p-GLP-2 and p-PYY) and III (p-GLP-2, p-PYY and p-OXM) after barley bread or legumes, respectively, was to some extent observed in the present work. However a parallel increase was only seen after BB(+)intervention period (p-GLP-1 and p-GLP-2). The discrepancy in p-GLP-2 response between the two BB intervention periods in the present paper remains to be elucidated.

P-glucagon levels increased at fasting following probiotic supplementation, compared to non-probiotic supplementation of the BB. However, the increase doesn't appear to have a major effect on b-glucose- and s-insulin concentrations, neither at fasting nor in the postprandial period. Glucagon acts counter-regulatory to insulin by stimulating hepatic output of glucose at hypoglycaemic conditions[227] and increased concentrations have been associated with T2D[228]. GLP-1 and GLP-2 are regulators of glucagon secretion. However their effects are opposite, with GLP-1 acting to suppress secretion[229] and GLP-2 to stimulate[230]. It has been suggested that the inhibitory effects of GLP-1 on glucagon release outweighs the stimulatory effects of GLP-2, resulting in functional glucagon suppression in response to a meal. The presently observed increase in both p-GLP-2 and p-glucagon following BB(+) compared with BB(-) is thus noteworthy, however the physiological relevance requires further investigations.

The glycaemic response following the standardized WWB breakfast was significantly decreased after BB(-) intervention period compared to WWB(-). No differences in

s-insulin response or measures of insulin sensitivity were observed depending on the preceding intervention period. Benefits on glycaemia have previously been established with barley kernel based products in an over-night perspective both here (Papers I and II) and previously[64, 153].

In the present study, additional risk markers were analyzed in comparison to Papers I, II and III. One such risk marker is PAI-1 which is considered to reflect endothelial function, and increased plasma levels of PAI-1 has been associated with T2D[231], CVD and systemic inflammation, especially triggered by LPS[232]. Further, PAI-1 is involved in the regulation of fibrinolysis and it has been hypothesized that disturbed fibrinolytic activity possibly provides a link between CVD and insulin resistance[233]. Dietary modulations resulting in decreased PAI-1 activity has been observed in healthy subjects after 2 weeks intake of high-NSP oat bran compared to low-NSP control. Further, probiotic treatment (*L. reuteri* GMNL-263) to pharmacologically induced diabetic rats during 28 days significantly reduced PAI-1 expression in the renal cortex and reduced glycaemia compared to diabetic control, with potential benefits in diabetic renal fibrosis[234]. In the present paper s-PAI-1 levels were increased at the standardized breakfast following BB(+) intervention period compared to BB(-) or WWB(-) intervention. The reason for this somewhat unexpected result remains to be clarified.

Taken together, the results indicates that metabolic benefits in terms of decreased glycaemia and increased concentrations of the antidiabetic, antiobesogenic hormone GLP-1 was observed after barley kernel based products without probiotic supplementation. Increased GLP-1 concentrations was also observed in the case of the BB supplemented with probiotics, however glycaemic benefits were absent. Instead, probiotic supplementation of BB increased levels of p-GLP-2 with potential benefits on gut barrier integrity, and increased p-glucagon and s-PAI-1. In general, there were no obvious benefits nor detrimental effects from inclusion of the specific probiotics included in the present study. The increase in p-glucagon and s-PAI-1 following probiotic supplementation needs to be further studied.

General discussion

The results from the present thesis presents novel insight to health benefits of high-DF, low-GI foods, as reported in epidemiological studies[15-17]. The foods studied, i.e. barley kernel based products, as well as legumes, are considered low-GI foods[55] with especially high content of intrinsic DF[188, 235], which taken together suggests interesting health potential in relation to common disorders such as T2D and CVD.

Glycaemic benefits of barley kernel products in the perspective from an evening meal to breakfast (10 h) is determined by the high-DF content of the late evening meal, rather than the low-GI[62, 153]. Other important characteristics include the distribution of RS, insoluble- and soluble NSP. Thus, boiled barley kernels induced superior blunting effects on glucose response in an over-night perspective compared to wheat kernels, despite comparable amount of total DF[153]. RS in food may be divided into different categories according to the features that constitutes its indigestibility; RS1 (physically/botanically inaccessible), RS2 (ungelatinized/native), RS3 (retrograded) and RS4 (chemically modified). Due to the botanical structure maintained in barley kernel based products, such foods can be anticipated to contain appreciable portions of RS1, and to some extent RS3. RS1 is not physically modified and once released from the NSP matrix during fermentation in the gut, RS1 can be expected to be rapidly available for microbial enzymes. Interestingly, it is not the NSP present in barley kernel products, nor the RS per se that can be assigned with over-night benefits, but rather the combination of the two substrates. Hence, supplementation of WWB with high amylose maize RS + barley NSP, in order to match the natural content of these components in barley kernel products, induced benefits on over-night glucose metabolism in healthy young subjects, whereas supplementation with barley NSP or RS only did not[63, 64, 152]. Moreover, other potentially bioactive components within the barley kernel products, such as vitamins, minerals and phytochemicals are unlikely to be responsible, judged from the absence of benefits on over-night glycaemia after evening meals with 100% WG barley flour porridges compared to WWB evening meals[63]; the WG barley flour product being essentially

devoid of RS compared to the intact kernel. Taken together, the specific combination of indigestible carbohydrates intrinsic to barley kernel based products promotes glycaemic benefits in an over-night perspective. The mechanisms are suggested to be related to gut fermentation of the indigestible carbohydrates in the barley kernel based products. The indigestible carbohydrates in the test products of the present thesis comprises different proportions of DF components i.e NSP and RS, but also of olicosaccharides. Legumes in particular are rich in raffinose-oligosaccharides[235]. Barley on the other hand is especially acknowledged for its high amounts of soluble, viscous β-glucans[188]. The increased concentration of gut metabolites, SCFA (Papers I-III) and breath H_2 (Papers I-IV), suggests that the indigestible carbohydrates, present in the test products, are able to promote increased gut micriobiota metabolism, i.e. fermentation. The fact that also brown beans may induce metabolic benefits in an over-night perspective, 10-14 h, is a novel observation.

Consequently, improved glycaemic regulation is present at a subsequent breakfast meal, preceded by barley kernel based products or boiled legumes, as a single evening meal or during 3-4 days prior to the experimental day. The decreased glycaemia in Papers II and III is accompanied also by decreased insulin responses. Different measures are available for assessing insulin sensitivity, where the euglycaemic clamp is considered the golden standard[236]. However clamps are time-consuming, costly and labor-intensive. Other indirect methods are available based on the oral glucose tolerance test. Yet, a procedure based on measurements of fasting and postprandial glucose- and insulin responses has been developed allowing for calculations of an insulin sensitivity index ($ISI_{composite}$)[144]. This procedure is a development of the homeostatic model assessment (HOMA) technique for insulin sensitivity measurements which includes only fasting values of glucose and insulin[237].

It has been reported that reduced insulin sensitivity was present already 13 years before onset of T2D[238]. Thus strategies to maintain or improve glucose tolerance and insulin sensitivity among healthy subjects appears as an important approach in the prevention of T2D. Interestingly, the present thesis observes facilitated blood glucose regulation in healthy young and middle-aged study groups after barley kernel- or legume products in an over-night perspective. In Paper I, lowered blood glucose was observed in a prolonged time perspective from 10.5-16 h following an evening meal with barley kernel products. A novel observation of specific importance is the improved glucose tolerance in an over-night perspective after barley kernel based evening meals when compared to a WWB also

among the older cohort as presented in Paper II. This subject group could be considered at risk for development of diabetes, due to the association between increased age and higher prevalence of diabetes[239]. The improved glucose tolerance was accompanied by an increase in insulin sensitivity as measured by $ISI_{composite}$. Similarly, increased $ISI_{composite}$ was seen in the younger study cohort after barley (Paper I) and BrB (Paper III) when compared to a WWB reference (Table 8).

Table 8. Insulin sensitivity index (ISIcomposite) at a subsequent breakfast after evening meals of barley kernel based products, brown beans, or reference WWB, respectively1.

Paper	WWB	Barley kernel based products	%[2]
I	0.070 ± 0.029[3, 4]	0.100 ± 0.038	42*
II	622 ± 66.2[5]	778 ± 84.9	25**
IV	651 ± 68.5[5]	713 ± 80.5	10
	WWB	**BrB**	
III	440 ± 34.3[4, 5]	567 ± 43.8	29**

[1]Values are presented as means ± SEM, *Different from WWB P < 0.05, ** P < 0.01. $ISI_{composite}$ are calculated as (10 000/squared root (fasting glucose × fasting insulin × iAUC 0-120 min glucose × iAUC 0-120 min insulin)). BrB, boiled brown beans; WWB, reference white wheat bread.

[2]Differences in concentrations of test variables after test products (barley kernel based or BrB, respectively) compared with reference WWB.

[3] Glucose mmol/L and Insulin pg/mL.

[4] Data not included in the publication.

[5] Glucose mmol/L and Insulin nmol/L.

Increased body weight is a major risk factor for the development of T2D and CVD[1, 30]. Several gut hormones display roles in appetite- and glucose regulation. The present thesis reports increased concentrations of satiety hormones p-GLP-1 and p-PYY after barley kernel based products, and increased levels of p-PYY, p-OXM and decreased concentrations of orexigenic p-ghrelin after BrB, in an over-night perspective in healthy subjects. Of specific interest is the observed increase in p-GLP-1, which was in parallel to a decrease in voluntary energy intake (-12%) at lunch, following an evening meal with BK compared to an evening meal with WWB (Paper I). In addition, inverse correlation between p-GLP-1 and ratings of hunger and desire to eat were reported. The observed decrease in energy intake is of the same magnitude as observed at a lunch meal, preceded by intravenous infusions of GLP-1 in healthy subjects[146]. The possibility to ameliorate metabolic responses with potential effects on actual food intake emerge as an especially interesting observation in relation to DF-rich products. The involvement of e.g. GLP-1 in regulation of glucose metabolism and appetite regulation is apparent from the literature, and recent data indicates a role of this hormone also in the attenuation of inflammation

in vitro[240, 241] and in T2D-subjects[242, 243]. It has been suggested that gut hormones, such as GLP-1, GLP-2 and PYY, constitutes a link between gut fermentation of indigestible carbohydrates and e.g. decreased glycaemia, lowered sub-clinical inflammation and reduced food intake based on studies in animal models[108, 205, 244-246]. Evidence of similar mechanisms appears to be present also in humans after intake of prebiotics (oligofructose)[95, 247]. The present thesis suggests that increased concentrations of SCFA and gut hormones, emerged from degradation of indigestible carbohydrates intrinsic to intact foods may act as mediators of metabolic benefits in healthy normal weight, young or elderly subjects. The potential link between gut fermentation and hormone release is supported by the positive correlation between total s-SCFA and p-PYY and p-GLP-2 in Paper II, and s-propionate and p-GLP-1 in Paper I.

It could be argued, that provided the over-all metabolic benefits on glucose regulation, insulin sensitivity and appetite regulation is caused by a mechanism related to gut fermentation; the simultaneous dietary provision of common probiotics might influence metabolic out-come. However, a dietary background including a mixture of commonly available probiotics did not affect overall glycaemia as observed after barley kernel based products in an over-night perspective compared with no probiotic (Paper IV). On the contrary, p-GLP-2 was increased which is potentially beneficial whereas the increase in p-glucagon and s-PAI-1 also seen in the probiotic background might be interpreted as negative, making it difficult to draw firm conclusions. Thus, the effects of the currently included prebiotics in combination with probiotics requires further investigations.

A novel observation in the present thesis is the increase of the gut-derived hormone GLP-2 in response to indigestible carbohydrates intrinsic to foods, i.e. barley kernel based- and legume products (Paper II-III). GLP-2 exerts effects on gut barrier integrity with potential benefits on sub-clinical inflammation[108]. No coherent results were observed between GLP-2 response and inflammatory responses to barley kernel products. However in Paper III markers of inflammation (s-IL-6 and s-IL-18) were reduced after an evening meal with BrB. The possible effects of legumes in modulating inflammation warrants further investigation. Interestingly, it was recently reported that a diet rich in barley kernel products and legumes (chickpeas and brown beans) significantly reduced overall cardiovascular risk score (Framingham 10-year risk) after 4 weeks in healthy, overweight women (50-72 years) compared to a nutritionally well-designed control diet.[248]. Thus, a potential preventive value in relation to CVD might be suggested by including barley kernel and legume products to the daily diet. In accordance, high intake of legumes was

related to decreased prevalence of T2D[249] and MetS[250], and a decreased risk of obesity[121]. Additionally, a combination of high intake of WG and beans reduced prevalence of insulin resistance in healthy Korean adults[251]. Thus, dietary regimens including specific DF-rich foods show protective potential in relation to MetS-related disorders. It cannot be disregarded that the presently studied barley kernel- and legume products by virtue of their low-GI features, and additional benefits related to gut fermentation of DF present, may provide more potent metabolic benefits than low-GI food devoid of fermentable carbohydrates.

Taken together, the results of the present work indicates metabolic advantages of DF-rich foods, i.e. barley based products and legumes, as judged from increased glucose tolerance, increased insulin sensitivity, beneficial effects on appetite regulatory hormones and decreased voluntary food intake. It is suggested that these benefits are mediated by gut fermentation and stimulation of gut hormones. Previously, the importance of cross-talk between the gut microbiota and host metabolism has been revealed in animal models focusing on inulin and fructo-oligosaccharides as prebiotic substrates in relation to obesity and related metabolic disorders[90, 105, 108, 149, 244]. A hypothetical illustration overviewing potential mechanisms for gut microbiota – host metabolism interactions, based on results of the present thesis is shown in Figure 4 (page 82). The mechanisms proposed may be partly responsible for the reduced prevalence of T2D and obesity, as reported in epidemiological studies with WG and legume diets. The results further provide a rational for exploiting combinations of prebiotic substrates aiming at reducing risk markers for MetS-related disorders. Thus supplementation with specific combinations of indigestible carbohydrates, as present in the barley- and legume kernel, opens new possibilities in tailoring of foods with additive health benefits. Moreover, the importance of a maintained botanical structure should be acknowledged, and might provide an important feature in addition to the WG concept. The results of the present thesis also add insights to be explored in the development of a next generation of low-GI foods, capable of facilitating blood glucose regulation at several consecutive meals.

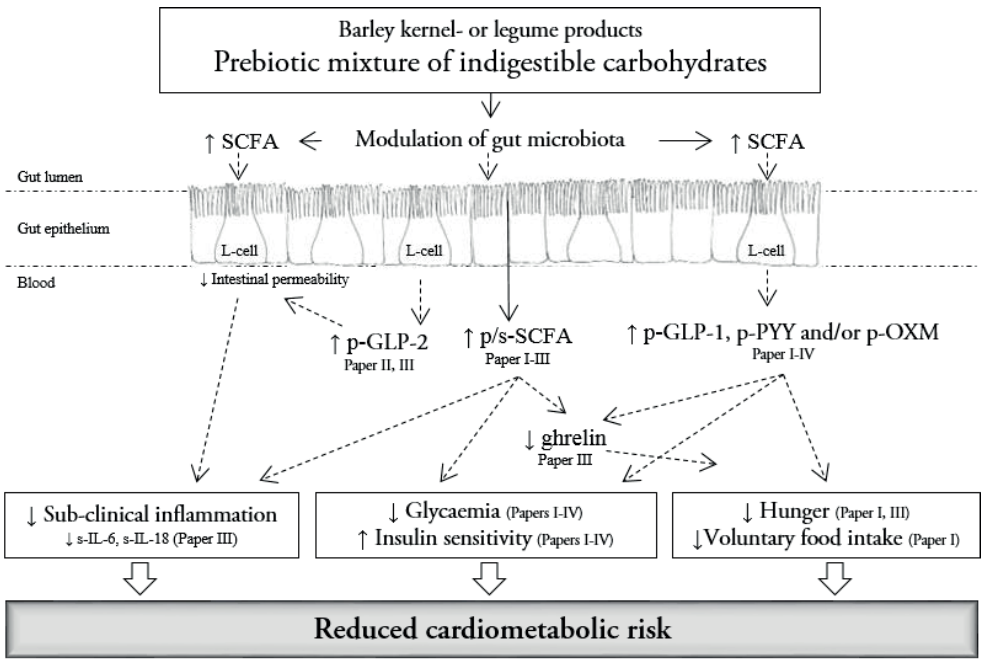

Figure 4. Hypothetical mechanisms for gut microbiota – host metabolism interactions.
Dotted arrows indicates suggested pathways.

Conclusions

The main conclusions drawn from the findings of the present work are given below.

- Improved glucose regulation was seen at breakfast after an evening meal with barley kernel products both in young adults and in middle-aged subjects. Intake of barley kernel based products decreased glycaemia for up to 10.5-16 h compared to WWB evening meal. Furthermore, in both the young and the more mature study groups, the improved glucose regulation was accompanied by increased insulin sensitivity ($ISI_{composite}$). In addition to barley kernels, brown beans possessed similar benefits.
- Several hormones important in glucose metabolism and appetite regulation were beneficially affected in an over-night perspective after barley kernel based products and/or brown beans, with increased gut hormones p-GLP-1, p-PYY, p-OXM and decreased p-ghrelin.
- Increased concentrations of p-GLP-2, with potential to improve gut barrier integrity were observed in the morning both after barley kernel based bread and brown bean evening meals, compared to WWB, with possible beneficial impact on inflammatory tonus.
- Intake of brown beans had the potential to reduce inflammatory markers (s-IL-6 and s-IL-18) in an over-night perspective.
- It was possible to reduce hunger sensations at breakfast preceded by brown beans or barley kernel based evening meals, compared to WWB. Decreased voluntary energy intake at lunch (-12%) was observed after an evening meal with boiled barley kernels.
- Inverse relations between the willingness to eat and gut hormones p-PYY and p-GLP-2, were detected in the morning after brown beans as an evening meal.

- Increased gut fermentative activity, as indicated from increased concentrations of breath H_2 and s-/p-SCFA were observed at a subsequent standardized breakfast after intake of barley kernels (total s-SCFA, s-propionate and (s-propionate + s-butyrate)) and brown beans (p-propionate and p-isobuturate) evening meals.
- Increased SCFA were related to increased levels of gut hormones. Consequently, a positive relation between p-propionate and p-GLP-1 was seen, and total s-SCFA were positively associated with p-PYY and p-GLP-2 at fasting after a barley kernel evening meal.
- A dietary background including a mixture of commonly available probiotics did not affect overall glycaemia as observed after barley kernel based products in an over-night perspective compared with no probiotic. On the contrary, p-GLP-2 was increased which is potentially beneficial whereas the increase in p-glucagon and s-PAI-1 also seen in the probiotic background might be interpreted as negative, making it difficult to draw firm conclusions.

The present thesis indicate metabolic advantages of DF-rich foods, such as barley based- and legume products, as judged from increased glucose tolerance, increased insulin sensitivity, decreased markers of inflammation, beneficial effects on appetite regulatory hormones and decreased voluntary food intake after approximately 10-14 h. The beneficial effects are suggested to emanate from mechanisms originating from gut fermentation of the indigestible substrates. Moreover, the importance of maintained botanical structure should be acknowledged, and might provide an important feature in addition to the WG concept.

Future perspective

An important focus for future studies would be to investigate the gut microbiota diversity and composition by microbiota analysis of faecal samples collected in healthy subjects provided barley kernel- or legume products using a similar experimental design as in the present work. Also, it would be relevant to explore possible added health benefits when combining the presently described DF-rich products in combination with bacterial strains appearing in faecic in higher abundance following ingestion of these foods.

Further studies may include different preparations of barley kernels, such as flaked or cut, in order to explore other possibilities to retain the specific DF distribution of the barley kernel based products. The different preparations may be administered in various products, such as bread, rice-analogues or breakfast cereals. Barley is available in different genotypes varying in carbohydrate content and structure. The potential of such barley genotypes should also be addressed with respect to their potential to affect glycaemic- and appetite regulating properties by stimulating gut hormones through a mechanism related to gut fermentation. Additionally, specific mixtures of indigestible carbohydrates intrinsic to the food products investigated could be evaluated for their potential as a food supplement.

Longer term intervention studies are further needed to confirm the present results in a broader cohort and in a "real-life" setting. Also of interest would be to study the impact of foods rich in specific DF on cardiometabolic risk factors in subjects with impaired glucose tolerance, overweight or obese, or subjects with overt T2D.

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