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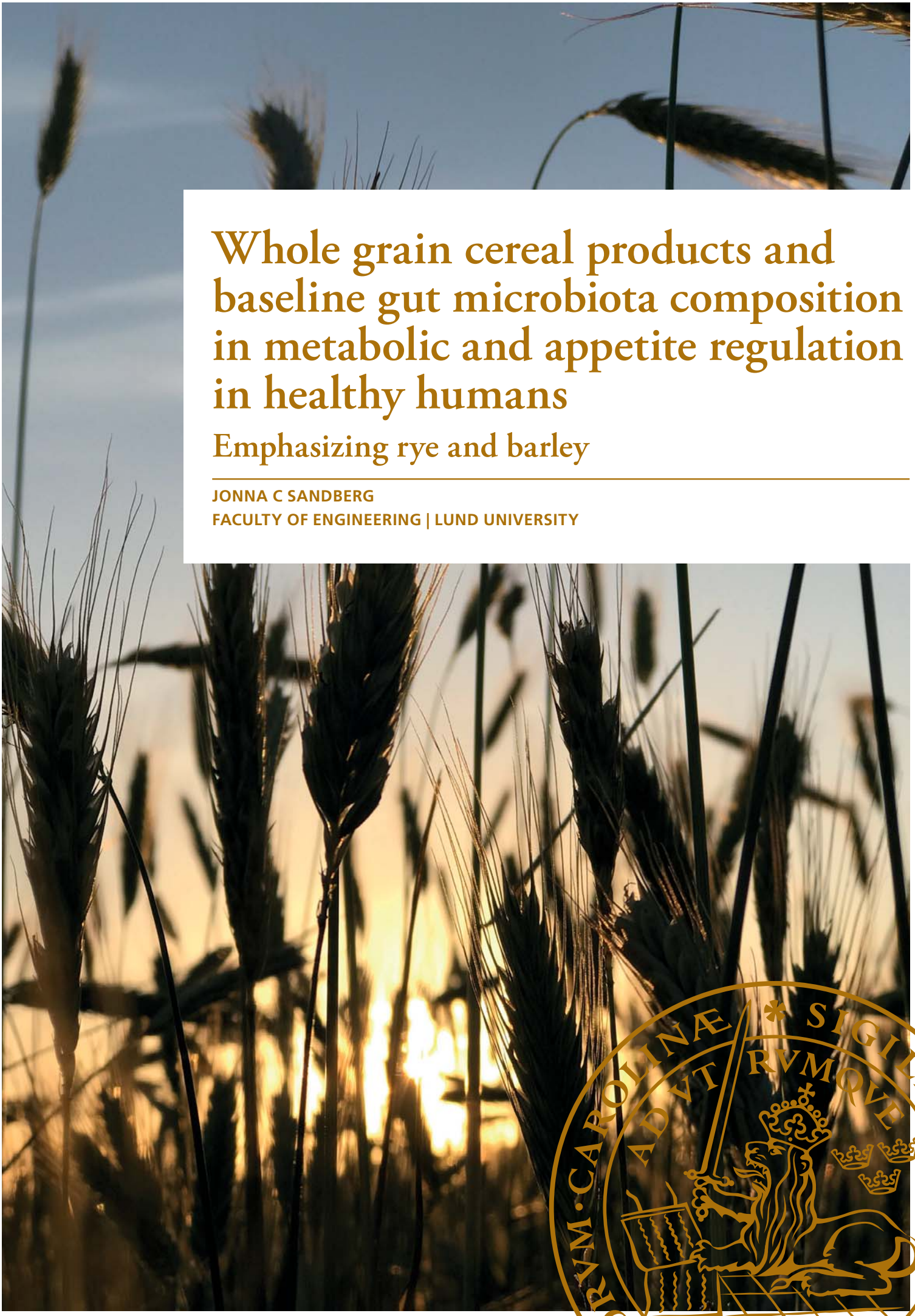

Whole grain cereal products and baseline gut microbiota composition in metabolic and appetite regulation in healthy humans

Emphasizing rye and barley

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LUND
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Title and subtitle **Whole grain cereal products and baseline gut microbiota composition in metabolic and appetite regulation in healthy humans - Emphasizing rye and barley**

Abstract

The prevalence of obesity, type 2 diabetes (T2D) and cardiovascular disease (CVD) is increasing continuously worldwide. These metabolic diseases are associated with many health issues, including increased risk of depression and cognitive impairment. Whole grain (WG) has shown potential to prevent obesity, T2D and CVD. One mechanism can be related to gut fermentation of specific indigestible carbohydrates, i.e. dietary fiber (DF).

The aim of this thesis was to investigate the relationship between gut fermentation of DF, and systemic effects including cardiometabolic risk markers and appetite regulation in healthy subjects. The thesis focuses on the potential prebiotic substrates in rye kernel and flour-based products, and the possible enhanced metabolic effects of adding commercial resistant starch (RS2) to rye flour. The thesis also undertook to evaluate effects of rye prebiotics on cognitive functions and/or mood. Furthermore, the impact of barley kernel products on metabolic regulation was studied in relation to gut abundance and ratios of *Prevotella* and *Bacteroides*. The thesis is based on four interventional studies with randomized crossover design, including healthy subjects of different ages (20–30 or 50–70 years). WG rye breads (based on kernels or WG rye flour or a WG rye kernel and flour mixture) or barley kernel-based breads, rich in DF, were provided as late evening meals. In addition, RS2 was added to some WG rye flour-based breads to substitute RS lost during the milling process. White wheat bread (WWB) was used as reference evening meal. In all studies, the subjects consumed the final portion of the test product in the evening (based on 50 g available carbohydrates), after which test variables were measured the next morning at fasting and following a standardized breakfast, i.e. 11–14 hours after ingestion of the test products.

In Paper I, a WG rye kernel-based (RKB) evening meal decreased the incremental glucose and insulin areas, and increased the appetite and glucose regulatory gut hormones GLP-1 and PYY, in young adults at a subsequent breakfast, compared to WWB. Furthermore, RKB increased total fasting SCFA (acetate, propionate and butyrate) concentrations and breath H_2 excretions the following day. In Paper II, effects on glucose, insulin and PYY concentrations could be further enhanced, in young adults, by replacing part of the rye kernels with WG rye flour and RS2. In Paper III, studies in middle-aged subjects showed that the WG rye mixture, in addition to its benefits on insulin sensitivity and PYY levels also increased GLP-2 concentrations, indicative of a beneficial effect on gut barrier function. This product also improved mood and increased feelings of happiness and wakefulness, compared to WWB. In Paper IV, the metabolic impact of a barley kernel product was studied in middle-aged subjects. The subjects were divided into different groups depending on gut *Prevotella*/*Bacteroides* ratio at baseline prior to the intervention. The results showed that the metabolic regulation differed between the test subjects depending on the baseline gut microbiota composition. Consequently, it was observed that a higher ratio of *Prevotella*/*Bacteroides* favored lower insulin response and inflammatory tone and subjective appetite ratings.

In summary, this thesis shows that WG rye products (rye kernel-based solely or blended with WG rye flour and RS2), may positively affect glucose and appetite regulation 11–14 h post ingestion. In addition, mood was improved post intake of the WG rye blend. The results indicate a prebiotic mechanism mediated via gut fermentation of DF in the WG rye breads. The studies also suggest that the metabolic regulation may be affected differently depending on gut microbiota composition. It is concluded that kernel-based rye or barley products as well as a blend of WG rye kernels/flour with RS have an anti-diabetic and anti-obesogenic potential.

Key words Obesity, type 2 diabetes, whole grain, rye, barley, gut microbiota, glucose regulation, gut fermentation

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Till min familj: ”Nu kör vi!”

Content

Abstract

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intervention. The results showed that the metabolic regulation differed between the test subjects depending on the baseline gut microbiota composition. Consequently, it was observed that a higher ratio of *Prevotella*/*Bacteroides* favored lower insulin response and inflammatory tone and subjective appetite ratings.

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Populärvetenskaplig sammanfattning

Denna avhandling visar att intag av fullkornsprodukter (bröd) baserade på rågkärnor har en positiv inverkan på blodsocker- och aptitreglering 11–14 timmar efter intaget, jämfört med fiberfattigt vitt bröd. Resultaten i avhandlingen visar dessutom att liknande effekter kan uppnås genom att ersätta en del av rågkärnorna med rågmjöl och därtill förstärka med fiberfraktion som gått förlorat under malningen, dvs resistent stärkelse (RS). Även humöret kan påverkas positivt genom intag av råg; försökspersoner som registrerade sitt humör i en humanstudie kände sig mer pigga och glada istället för slöa och nedslagna efter råg jämfört med vitt bröd. De gynnsamma effekterna av råg kan kopplas till tjocktarmsfermentering (tarmbakteriers nedbrytning av kostfiber) av den specifika kostfiberblandningen (”prebiotika”) som finns i råg. Man vet idag att tarmfloran och dess aktiviteter har stor betydelse för reglering av ämnesomsättningen. I avhandlingsarbetet observerades att tarmfloran hos friska försökspersoner hade en inverkan på individuell insulinfrisättning efter måltid, samt på inflammationsmarkörer och aptit. Ovan nämnda effekter av råg samt observationerna angående tarmflorans betydelse för reglering av ämnesomsättningen hos friska personer är helt ny kunskap och har stor betydelse eftersom den ökar förståelsen för hur kostfiberrika fullkornsprodukter och förändringar av tarmfloran kan användas i förebyggandet av fetma, diabetes typ 2 (T2D) och hjärtkärlsjukdomar.

Avhandlingen baseras på fyra koststudier som inkluderade friska personer i olika åldrar (20-30 eller 50-70 år). Anledningen till att studierna endast inkluderade friska personer var för att kunna påvisa hur fullkornsprodukter kan underlätta förebyggandet av metabola sjukdomar såsom fetma och T2D. I samtliga studier konsumerade försökspersonerna den sista portionen av testprodukten på kvällen, varpå de anlände till försöksavdelningen nästa morgon för provtagningar. Anledningen till att testprodukterna intogs på kvällen var för att kunna säkerställa att kostfiberkomponenterna hade hunnit nå tjocktarmen och att man därför kunde förutsätta att eventuella effekter på testmarkörerna härrörde från tjocktarms-fermentering av kostfibrerna i råg. Kostfibrerna fungerar som näring åt bakterierna, och då bakterierna fermenterar kostfibrerna bildas bland annat kortkedjiga fettsyror (SCFA), som anses ha flera hälsosamma effekter kopplade till tarm och ämnesomsättningen; exempelvis underlättas frisättning av tarmhormonerna GLP-1 och PYY i tarmen, vilka spelar en viktig roll i glukos- och aptitregleringen.

Bakgrunden till avhandlingen är den ökande förekomsten av livsstilsrelaterade hälsoproblem i världen såsom fetma, T2D och hjärtkärlsjukdomar. Fetma ökar risken att utveckla T2D som i sin tur medför ökad risk för hjärtkärlsjukdom och dödlighet, men har också kopplats till en ökad risk för depression och kognitiv

nedsättning. Eftersom dessa hälsoproblem är starkt relaterade till livsstilen, t ex fysisk aktivitet och diet, innebär det att de faktiskt går att förebygga. Fullkorn har länge ansetts vara en del av en hälsosam diet, och studier har visat att fullkorn har positiva effekter vid förebyggandet av bl a T2D och hjärtkärlsjukdomar. En viktig del i förebyggandet av dessa metabola sjukdomar är att främja konsumtion av livsmedel som resulterar i låga blodsockerpåslag efter måltider, eftersom höga blodsockertoppar tröttar ut insulinbildande celler och ökar oxidativ stress och inflammation, vilket ökar risken för metabola sjukdomar. Rågprodukter resulterar i ett jämnt och lågt glukossvar efter en måltid, dvs har ett lågt glykemiskt index (GI), men som visats i denna avhandling påverkar även rågprodukter glukostoleransen gynnsamt 11-14 timmar efter intag.

En ökad mättnadskänsla och en minskad inflammation är fördelaktigt i förebyggandet av fetma (”anti-fetma”-egenskaper). Rågbröd bestående av hela rågkärnor resulterade i en ökning av tarmhormonerna GLP-1 och PYY, även kallade mättnadshormoner. Försökspersonerna kände sig dessutom mättare efter intag. Vidare studerades effekter av råg då en del av rågkärnorna ersattes med rågmjöl samt RS (RB+RS) eftersom malning av cerealiekärnor till mjöl medför att en del kostfiber i form av RS försvinner. Efter intag av RB+RS ökade PYY och även ett annat tarmhormon: GLP-2. GLP-2 bidrar till antiinflammatorisk effekt genom att förstärka tarmväggens barriär så att inflammationsstimulerande molekyler inte ”läcker” genom tarmen och tar sig in i blodbanan.

Det finns idag en ökad insikt beträffande tarmflorans roll i glukosreglering och aptitkontroll. Till exempel har man sett att tarmfloran är annorlunda hos friska människor jämfört med T2D patienter. Dock kan tarmfloran även variera mellan friska personer, och i en av studierna i avhandlingen delades försökspersonerna in i olika grupper beroende på deras tarmflora. Resultaten visade att t ex insulinfrisättning efter måltid, inflammationsnivå och aptitkänslor skilde sig åt mellan försökspersonerna beroende på sammansättningen av tarmfloran.

Sammanfattningsvis visar denna avhandling att genom att äta kärnbaserade rågbröd samt rågmjölsbröd kompenserade med fiber i form av RS kan man erhålla positiva effekter på glukos- och aptitreglering upp till en halv dag efter intag (ca 14 timmar). Resultaten från avhandlingen indikerar att troliga mekanismer involverar tjocktarmsfermentering av kostfiber i råg. Studierna visar även att tarmfloran har betydelse för vår metabola reglering. Slutligen, studierna har påvisat en antidiabetisk och ”anti-fetma”-förmåga hos råg som kan vara fördelaktigt i förebyggandet av fetma, diabetes typ 2 och hjärtkärlsjukdomar.

List of Papers

This thesis is based on the following papers:

Paper I **Rye-based evening meals favorably affected glucose regulation and appetite variables at the following breakfast; a randomized controlled study in healthy subjects**

J. C. Sandberg, I. M. E. Björck, A. C. Nilsson
PLoS ONE 2016 11(3): e0151985

Paper II **Effects of whole grain rye, with and without resistant starch type 2 supplementation, on glucose tolerance, gut hormones, inflammation and appetite regulation in an 11–14.5 hour perspective; a randomized controlled study in healthy subjects**

J. C. Sandberg, I. M. E. Björck, A. C. Nilsson
Nutrition Journal 2017 16(1):25

Paper III **Impact of rye-based evening meals on cardiometabolic risk factors, cognitive functions and mood in healthy middle-aged subjects**

J. C. Sandberg, I. M. E. Björck, A. C. Nilsson
Submitted Manuscript

Paper IV **Abundance of gut *Prevotella* at baseline and metabolic response to barley prebiotics**

J. Sandberg, P. Kovatcheva-Datchary, I. Björck, F. Bäckhed, A. Nilsson
Submitted Manuscript

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The Author's Contributions

Paper I

The author, J. Sandberg, took part in study design, coordinated the study, recruited and screened participants, performed the experimental work and the statistical calculations, took part in evaluating the data and writing the manuscript.

Paper II

The author, J. Sandberg, was involved in study design, coordinated the study, recruited and screened participants, performed the majority of the experimental work and the statistical calculations, took part in evaluating the data and writing the manuscript.

Paper III

The author, J. Sandberg, was responsible for the study design regarding metabolic aspects, coordinated the study, took part in aspects regarding cognitive study design, and was responsible for oral cognitive testing, the analysis and evaluation of the results and for writing the manuscript.

Paper IV

The author, J. Sandberg, was involved in the study design, coordinated the study, performed the majority of the experimental work, and was responsible for analysis and statistical evaluation of the metabolic test variables and for writing the manuscript.

Abbreviations

AX	arabinoxylans
b	whole blood
BDNF	brain-derived neurotrophic factor
BKB	barley kernel-based bread
BMI	body mass index
CRP	c-reactive protein
CVD	cardiovascular disease
DF	dietary fiber
dm	dry matter
FFA	free fatty acids
GI	glycemic index
GLP	glucagon-like peptide
HOMA-IR	homeostatic model assessment of insulin resistance
HP	high *Prevotella*
HPB	high *Prevotella* and *Bacteroides*
(i)AUC	(incremental) area under the curve
(i)Peak	maximum postprandial increase from baseline
IL	interleukin
$ISI_{composite}$	insulin sensitivity index
LP	low *Prevotella*
LPS	lipopolysaccharide
LPB	lipopolysaccharide binding protein
MetS	metabolic syndrome
NSP	non-starch polysaccharides
p	plasma
PYY	peptide YY
PAI	plasminogen activator inhibitor

RFB	rye flour-based bread
RKB	rye kernel-based bread
RS	resistant starch
SA	selective attention
SCFA	short chain fatty acids
s	serum
T2D	type 2 diabetes
VAS	visual analogue scale
WG	whole grain
WM	working memory
WWB	white wheat bread

Introduction

In 2014, more than 1.9 billion people over the age of 18 were overweight, including the 600 million people who were classified as obese [1]. This means that more than one third of the global population is overweight, and 1 out of 10 people are obese. Obesity is a major risk factor for developing a range of diseases including those linked to the metabolic syndrome (MetS), e.g. cardiovascular disease (CVD) and type 2 diabetes (T2D). Obesity is associated with insulin resistance, a state where the sensitivity to insulin in the tissues is decreased and more insulin is needed to obtain a normal glucose tolerance. Development of insulin resistance is suggested to be due to obesity-induced low-grade inflammation [2]. Furthermore, obesity and the MetS also predispose for depression and cognitive decline, including dementia and Alzheimer's disease [3]. Thus, Alzheimer's disease has displayed connections both to dysfunction in glucose metabolism and insulin signaling in the brain.

The cause of the increase in the prevalence of obesity and T2D is multi-factorial. However, poor diet quality, high accessibility to energy-dense foods and low physical activity are important contributory factors. Consequently, strategies for primary prevention of obesity and T2D are needed, addressing life-style, including measures to increase physical activity or improve diet quality. In this context, dietary changes towards an increased intake of whole grain (WG) and dietary fiber (DF) have shown promising results. Epidemiological studies have thus shown that DF intake is associated with a reduced risk of CVD [4], T2D [5] and prevention of body weight gain [6]. The preventive mechanisms of WG are not fully understood but important factors may include a reduction of oxidative stress and systemic low-grade inflammation by maintaining a tighter glucose regulation [7]. Furthermore, WG is a rich source of DF, and colonic fermentation of DF has been suggested as a likely contributory factor to the beneficial effects shown by WG. Consequently, more recently, it has been suggested that the composition of gut microbiota probably has a significant impact on metabolic regulation of peripheral tissue [8]. Accordingly, it has been observed that the composition of the gut microbiota is different in individuals with MetS compared to healthy individuals. In addition, it has been suggested that gut microbiota have an impact on cognition and mood via the gut-brain axis [9, 10], which i. a bidirectional communication between the gut and the brain.

Acute postprandial response of glucose and insulin to WG has been widely studied. In this respect, both WG barley and rye kernels have displayed low glycemic index (GI) character. Previous work has shown that barley kernel-based evening meals, rich in DF, induce beneficial impact on glucose and insulin responses as well as on appetite regulation following a standardized breakfast, ingested 11–16 h after the barley evening meal [11]. The effects in this longer, semi-acute time perspective are suggested to emanate from colonic fermentation of DF included in the cereals. Rye-based products have previously been shown to have beneficial postprandial effects on glucose regulation (0-120 min post intake) and appetite sensations 4.5 h after intake [12]. However, less is known about the potential metabolic benefits of rye in a longer, semi-acute time perspective, for example 11–14 h after intake. Interestingly, recent work has shown that the benefits from, for example, barley DF on glucose tolerance differ depending on the gut microbiota composition [13]. Therefore, it is of interest not only to investigate metabolic effects of WG food on group mean basis, but also to investigate differences based on individual gut microbiota composition.

The present thesis aims to elucidate the effect of rye on cardiometabolic risk factors and to investigate effects on memory functions and mood in healthy subjects, focusing on the impact of gut fermentation. In addition, the thesis aims to elucidate the impact of baseline gut microbiota composition in metabolic regulation, focusing on the baseline ratio of *Prevotella*/*Bacteroides*.

The work presented in this thesis includes four intervention studies in healthy volunteer subjects, all performed in a semi-acute overnight perspective, i.e. test products are provided in the evening and test variables determined the next morning after an overnight fast.

Background

Obesity and the Metabolic Syndrome

Obesity is characterized by excessive fat accumulation caused by an energy imbalance between energy intake and energy expenditure. Different measures of obesity have been introduced, e.g. body mass index (BMI), waist circumference and waist-to-hip ratio, with the most commonly adopted being the BMI. Adults with a BMI above 30 kg/m^2, are regarded as obese [1]. Obesity is associated with an increased risk of metabolic abnormalities, including T2D and CVD [1]. Thus, obesity is linked to the development of elevated blood pressure, triglycerides, fasting glucose and a pro-inflammatory state, and lower HDL concentrations [14].

The definition of the metabolic syndrome (MetS) varies in guidelines; some definitions are more focused on insulin resistance as a main criterion, whereas other definitions regard obesity (waist circumference) to be a central criterion in the MetS definition [15]. Moreover, a pro-inflammatory state is regarded as an additional metabolic criterion of MetS diagnostics by, for example, the International Diabetes Federation (IDF) [16]. In 2009, Alberti et al. published a consensus definition of the MetS and the criteria for diagnosis of MetS included fulfillment of any three of the five following risk factors: elevated waist circumference, triglycerides, blood pressure and fasting blood glucose, and reduced HDL cholesterol. See **Table 1** for cut-off values [14]. Consequently, a strong association is observed between obesity and MetS based on similarities between the obesity-related complications and the metabolic risk factors of MetS [17]. It has been shown that MetS is associated with twice the risk of developing CVD [18] and five times the risk of developing T2D [19]. Furthermore, MetS has been shown to increase the risk of cognitive decline and the development of Alzheimer's disease [3].

Table 1.
Diagnostic criteria for the MetS, including fulfillment of any 3 out of 5 risk factors, according to Alberti et al. 2009 [14].

Risk factors	Cut points
Elevated waist circumference*	≥94 cm (males) and ≥80 cm (females)
Elevated triglycerides	≥150 mg/dL
Reduced HDL-C	<40 mg/dL (males) and <50 mg/dL (females)
Elevated blood pressure	Systolic ≥130 and/or diastolic ≥85 mm Hg
Elevated fasting glucose	≥5.6 mmol/L

*People of European origin (population specific definitions) according to IDF [16].

Low-grade Inflammation

A state of increased low-grade chronic inflammation is associated with obesity and is a recognized pathological feature of T2D and CVD [20, 21]. Low-grade inflammation involves increased levels of cytokines e.g. IL-1β, IL-6 and TNF-α, which can interfere with insulin signaling and lead to insulin resistance [22]. Studies have suggested that there is a relationship between inflammation and diet. Thus, foods such as fats, processed meat and refined grains have been positively associated with inflammatory markers, whereas a negative association has been observed between inflammatory markers and whole grain, fruit and nuts [23, 24].

It has been shown that acute hyperglycemia spikes can cause a pro-inflammatory response, indicating that a lower rate of glucose into the bloodstream would be preferred. Consequently, rapid elevations in blood glucose concentrations have been associated with increased levels of IL-6 [25], which in turn are mediated by hyperglycemia-induced oxidative stress [26]. Thus, the promoted low-grade inflammation by hyperglycemia demonstrates a link between T2D and CVD. Systemic inflammation may also impair central insulin signaling, hence impeding memory functions, as insulin in the central nervous system has been shown to play a key role in memory and learning [27].

It has also been suggested that increased low-grade chronic inflammation in metabolic diseases may in part be mediated through modulation of the gut microbiota via high-fat feeding [28], see below in the section “Gut Microbiota and Metabolism”.

Glycemic Index and Glucose Regulation

The glycemic index (GI) provides a measure of the quality of carbohydrates in a food by comparing the acute blood glucose response after intake of the food item with a standard that usually consists of a glucose solution or a white bread. The

refined white bread is regarded as a high GI food that is rapidly digested and absorbed, and quickly raises the blood glucose levels. On the contrary, a low GI food will promote a slow rate of glucose release into the bloodstream. Epidemiological studies have shown that low GI foods decrease the risk of T2D and CVD [29, 30]. Low GI foods have also displayed beneficial effects on cognitive performance in healthy subjects [31] and in patients with T2D [32].

There are several factors affecting the acute glucose response of starchy food items, and one of them is the botanical structure, e.g. intact botanical structure versus flour of the same grain. Consequently, it has been shown that intact barley kernels lowered the postprandial glucose response, while no such effects were observed when milling the kernels to flour and serving it as porridge [33]. In addition, some whole grain (WG) foods have been shown to improve the postprandial glucose response at a subsequent meal; an effect called the "second meal effect". The time interval between the first and second meal can be around 4 hours (e.g. breakfast to lunch) or up to 11 hours (e.g. dinner to breakfast). The mechanisms mediating the beneficial effects on blood glucose are different depending on the time interval. The improved glucose response in a 4-hours perspective is suggested to depend on low GI characteristics *per se*, resulting in suppressed free fatty acids (FFA) and, thus, increased insulin sensitivity. However, whereas a low-GI *per se* is responsible for a beneficial effect on postprandial glucose regulation in a 4-hour second meal perspective, the improved glucose tolerance seen approximately 11 hours after intake of certain WG products is more likely instead to be mediated by the presence of specific DF mixtures that are fermented in the colon [34]. Consequently, not all low-GI products result in a beneficial effect on glucose tolerance in this prolonged time interval. Furthermore, it has been shown that similar benefits on glucose tolerance seen after 11 hours post intake of barley kernel-based products can be obtained by mimicking the DF composition in barley kernels using barley DF and adding resistant starch (RS) to a white wheat flour bread. However, no beneficial effects on glucose regulation were noticed when adding DF or RS separately to the flour-based bread [34] [33], indicating the importance of the combined DF and RS found in intact barley kernels.

Whole Grain Products and their Role in Health

Whole grain is regarded as part of a healthy diet and has been shown to be associated with a reduced risk of obesity [4], type 2 diabetes [35] and cardiovascular disease [36]. WG is rich in dietary fiber (DF) and, according to the Nordic nutrition recommendation (NNR 2012), the recommendations for the daily

intake of WG and DF are 70–90 g and 25–35 g, respectively [37], but the recommendations are seldom reached. This is problematic as DF, like WG, is regarded to have preventive properties against metabolic disorders [6, 38]. The DF in WG cereals are mainly located in the outer layer of the grain. Thus, much of the DF is lost in the refined version of cereals such as refined white wheat flour. The DF components include non-starch polysaccharides (NSP), RS, oligosaccharides (mainly fructans) and lignin. The main NSP are arabinoxylans (AX), β-glucans and cellulose [39]. The NSP consist of soluble and insoluble fibers, while there are four different categories of RS (RS1-RS4). The main RS in intact WG kernels belongs to category RS1, which is starch entrapped in the non-digestible matrix. The amount of RS1 depends on the physical inaccessibility, i.e. RS1 is higher in intact kernels and is minimized by milling. RS2 includes ungelatinized starch from e.g. raw potatoes and high-amylose maize-starch. The RS3 and RS4 comprise retrograded and chemically modified starch, respectively [40]. WG is also a rich source of other bioactive components such as micronutrients (e.g. vitamins and minerals) and phytochemicals (e.g. phytic acid and phenolic acid), which have been associated with several protective effects such as improving insulin sensitivity and providing antioxidant protection [41].

WG cereals such as WG rye and WG barley are rich sources of DF. Rye (*Secale cereale* L.) is one of the most commonly consumed WG cereals in Scandinavia [42]. Studies including rye have previously been shown to improve glucose and appetite regulation in healthy subjects. Thus, intake of rye kernels for breakfast has been shown to lower early glucose response, decrease insulin response, increase acute subjective feelings of satiety and decrease energy intake at lunch [12]. In addition, rye kernel intake has displayed sustained satiety feelings during prolonged ingestion [43]. Furthermore, intake of WG rye kernels for breakfast has been shown to induce lower daylong glucose response and increase breath hydrogen (H_2) excretion, indicating increased colonic activity [33]. However, studies investigating the metabolic effects of fermentation of dietary fiber in rye are scarce. Barley (*Hordeum vulgare* L.) is a cereal that is less commonly consumed in Scandinavian countries, compared to rye-based products. However, barley-based products have displayed beneficial effects on cardiometabolic risk markers and appetite regulation, both in an acute and semi-acute (4–6 h and 10–14 h after intake) perspective [11, 33, 44].

Definition of WG and DF

The definition of WG varies slightly between areas in the world. In the EU project HEALTHGRAIN whole grain was defined as: “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" [45]. Thus, the WG definition includes both kernels and recombined milled fractions (wholemeal). Recently, the HEALTHGRAIN Consortium also suggested the following definition for *whole-grain foods*: "Whole-grain food is one for which the product is made with ≥30% whole-grain ingredients on a dry-weight basis and more whole-grain ingredients than refined-grain ingredients" [46]. Consequently, the definition was suggested to facilitate for the consumers to choose foods with high WG content.

There are several definitions of DF and the definition of DF according to CODEX Alimentarius Commission 2009 is: "Dietary fiber means carbohydrate polymers with ten or more monomeric units, which are not hydrolyzed by the endogenous enzymes in the small intestine" [47]. Indigestible carbohydrates with DP 3–9 are thus not included in the definition; instead, inclusion should be decided by national authorities. Consequently, authorities such as the European Commission and the American Association of Cereal Chemists have both decided to include indigestible carbohydrates with DP of 3–9 in the DF definition [48, 49].

The definition of prebiotic was recently (published online in June 2017) updated by International Scientific Association for Probiotics and Prebiotics as followed: "A substrate that is selectively utilized by host microorganisms conferring a health benefit" [50]. The new definition recognizes that dietary prebiotics can promote health benefits by stimulation of beneficial taxa in addition to bifidobacteria and lactobacilli, which have previously been mostly referred to regarding selective stimulation. Currently, fructans and galactans are the dietary prebiotics that have been most extensively documented regarding health benefits in humans [50].

Gut Microbiota Fermentation and Host Metabolism

The number of bacterial cells in the human microbiota is estimated to be as much as 10^{14} bacterial cells. The gastrointestinal tract is the human organ colonized by the most microbiota, which is unevenly distributed along the gastrointestinal tract, with the highest abundance in the large intestine, i.e. around 70% of the total amount of microbes in the human body [51]. The human gut is mainly colonized by the phyla Firmicutes and Bacteroidetes, whereas other bacterial phyla including Proteobacteria, Verrucomicrobia, Actinobacteria, Fusobacteria and Cyanobacteria are present in minor proportions [52]. The gut microbiota is involved in several aspects of importance for the host's health, e.g. metabolism of indigestible carbohydrates, impact on energy harvest from ingested food, regulation of host metabolism and production of vitamins [51]. Furthermore, gut microbiota contributes to the maintenance of intestinal epithelial barrier function, and in the

development and maturation of intestinal mucosal and systemic immune system [51]. Diet is regarded as a key factor in determining the composition of gut microbiota [53]. Other examples of host-dependent factors are general lifestyle, genetic background, early colonization and medication. Studies focusing on influences of diet habits on the gut microbiota composition show that gut abundance of *Prevotella* and *Xylanibacter* is higher when the diet is characterized by high amounts of DF, whereas a diet consisting of a high intake of animal protein and fat, and low in DF, i.e. a typical western diet, is instead shown to increase the abundance of *Bacteroides* [54, 55]. Recent studies have shown that not all subjects display similar beneficial metabolic responses, such as on glucose tolerance or weight loss, to meals or more specific food items although they are identical. It has been suggested that the underlying mechanisms to the discrepancy in metabolic effects are connected to differences in the gut microbiota composition [13, 56-58].

Gut Fermentation of Dietary Fibers

An essential role of the gut microbiota in the large intestine is the ability to ferment DF. During fermentation, metabolites are formed, such as short chain fatty acids (SCFA) including acetate, propionate and butyrate, and also gases, such as carbon dioxide, hydrogen and methane [59]. Succinate is another acid formed during fermentation of DF and is mostly considered as an intermediate for further conversion to propionate [60], but lately it has been suggested that succinate may have important signal functions, for example with respect to glucose homeostasis [61]. Most of the SCFA produced during fermentation is either absorbed by the colonic epithelium or used as nutrient by other bacteria in the gut, resulting in a remaining SCFA content in feces of only around 5–10% of that originally produced [39]. A major part of the remaining SCFA enters the portal vein and is used by the liver for cholesterol synthesizing [62]. However, a minor part of the SCFA enters circulation, reaching the peripheral tissue, and has shown beneficial metabolic effects, e.g. increasing insulin sensitivity [63]. Suggested metabolic pathways and effects are shown in **Figure 1**. SCFA are involved in positive effects regarding colonic health, such as acting as an energy source for the epithelium cells and decreasing gut permeability, and have been shown to have anti-inflammatory properties in the colon [64]. SCFA have also been shown to bind to the free fatty acids receptors (FFAR) 2 (formerly known as GPR43) and FFAR3 (formerly known as GPR41) present on colonic L-cells, promoting secretion of gut hormones, e.g. the satiety-regulatory hormones GLP-1 and PYY, involved in the gut-brain axis (see the section “Gut-Brain Axis”) [65, 66].

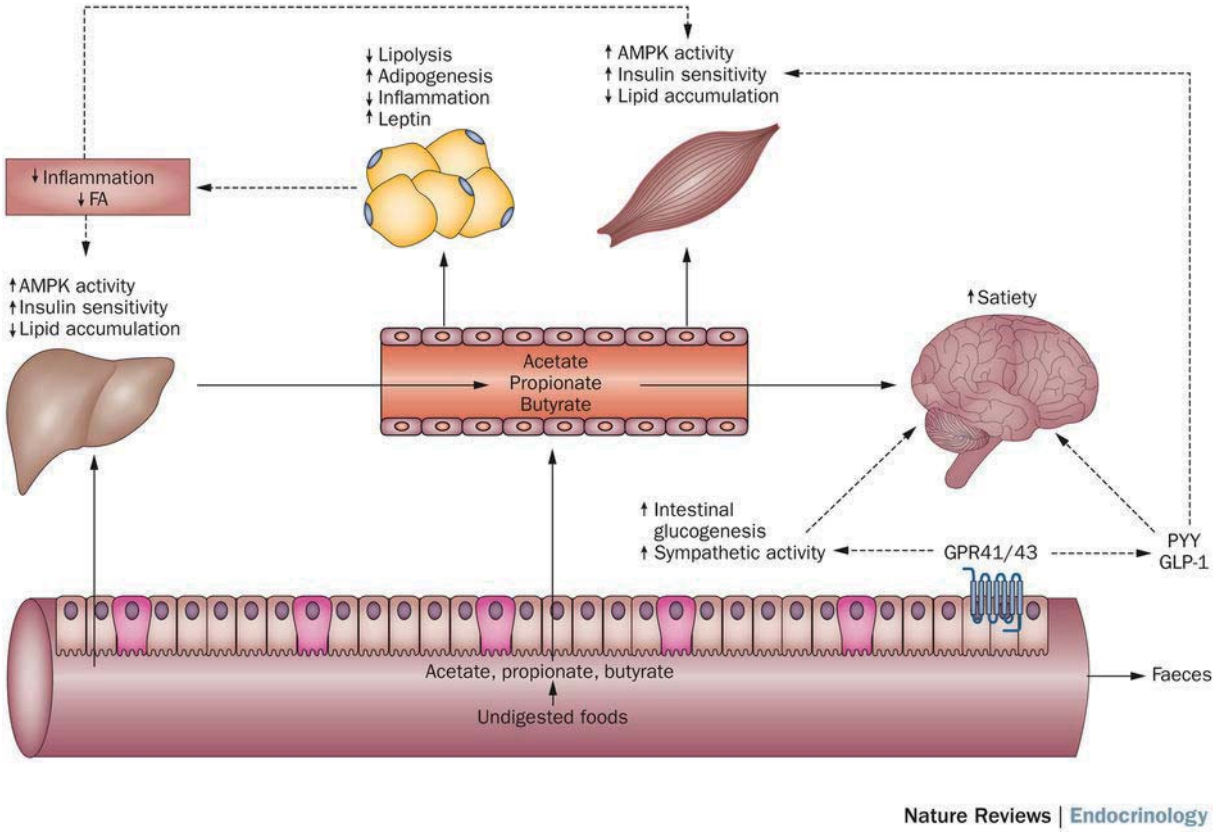

Figure 1. SCFA and interorgan crosstalk.
Reprinted from Nature Reviews Endocrinology, 11/10, Canfora, E. E., Jocken, J. W., & Blaak, E. E., Short-chain fatty acids in control of body weight and insulin sensitivity, p 577-591, with permission from Nature Publishing Group.

Gut Microbiota and Metabolism

There is a growing insight regarding the alterations of the gut microbiota composition and metabolic diseases including obesity and type 2 diabetes [8]. Obesity has been associated with alterations of the Bacteroidetes/Firmicutes ratio, displaying an increase in Firmicutes [67]. Consequently, these alterations have been associated with increased capacity to harvest energy from the diet, which increases the absorption of calories. The relation between gut microbiota composition in obese subjects and the degree of adiposity has also been shown in a mouse model by transferring caecal microbiota from an obese mouse to a germ-free normal-weight mouse. This resulted in a significant increase in adiposity in the germ-free mouse, compared to a germ-free normal-weight mouse transplanted with microbiota from a lean mouse [68]. In patients with T2D, a dysbiosis in gut microbiota was observed including a decline in butyrate-producing bacteria, e.g. *Faecalibacterium prausnitzii*, and an increase in opportunistic pathogenes, e.g. *Bacteroides caccae* [69].

Lipopolysaccharide (LPS) is present in the cell wall of Gram negative bacteria and is considered an endotoxin. Metabolic endotoxemia was defined by Cani et al as the increase in plasma LPS induced by high-fat feeding [28]. Thus, the high-fat diet alters the gut microbiota composition towards increased production of LPS, and increases gut permeability by altering the protective functions of the gut epithelium [70]. As mentioned above, obesity and insulin resistance are associated with chronic low-grade systemic inflammation. One hypothesis regarding possible underlying mechanisms for the association between the gut microbiota and the metabolism of the host relates to metabolic endotoxemia [28]. Development of metabolic endotoxemia is connected with increased concentrations of LPS in plasma, resulting in a low-grade systemic inflammation by stimulating the innate immune response to LPS and production of cytokines, which in turn leads to increased insulin resistance.

Gut Hormones and the Gut-Brain Axis

The bidirectional communication between the gastrointestinal tract and the brain, known as the gut-brain axis, is composed of neuronal, endocrine and immune-related signaling and involves several pathways affecting the glucose and energy homeostasis, and inflammation [71]. Gut hormones produced in the gastrointestinal tract are importantly involved in the regulation of metabolism and appetite. It has been shown that the gut hormones communicate with appetite-regulating regions within the central nervous system through the gut-brain axis. The gut hormones either signal satiety, (GLP-1, PYY and OXM) or hunger (ghrelin), to these brain areas via the vagus nerve or via blood circulation. GLP-1, PYY and OXM are produced in L-cells located in the ileum and colon, and are secreted proportionally in response to nutrient intake. The satiating effect of GLP-1, PYY and OXM has been evaluated in human interventions showing reduced food intake after peripheral infusion of the hormones [72]. In addition to promoting satiety, GLP-1 is recognized as an incretin based on its ability to enhance insulin secretion. GLP-1 is also a mediator of the ileal break where the gastric emptying rate is reduced, resulting in improved glucose tolerance and decreased insulin concentration due to delayed carbohydrate absorption [73]. Consequently, GLP-1 analogues are currently used as a treatment for T2D patients [74]. GLP-1 has also displayed involvement in cognitive functions by displaying effects on neuroprotection and learning [75]. Additionally the satiety-signaling gut hormone PYY is suggested to improve insulin sensitivity [76]. Ghrelin is mainly produced by the epithelial cells in the stomach and is a hormone that elicits orexigenic effects. Thus, contrary to GLP-1 and PYY, ghrelin has been shown to stimulate food intake in humans [72].

Objective

The prevalence of obesity and related disorders are increasing worldwide. The main objective of this thesis was to evaluate the metabolic potential of indigestible carbohydrates present in certain WG cereal products, with focus on glucose metabolism, appetite regulation, cognitive performance and mood parameters. Metabolic effects were evaluated in healthy volunteers in semi-acute studies; test products being ingested in the evening and test variables followed at a subsequent standardized breakfast. Test products were WG bread made from rye or barley kernels. In the case of rye, breads were also made from 100% WG rye flour or a blend of kernels and WG rye flour. The breads including rye flour were in addition evaluated after supplementation of the flour with commercial resistant starch (RS) to mimic the indigestible carbohydrate profile in kernel-based rye bread. In the case of barley kernel bread, the impact of the gut microbiota composition on host metabolism was also studied, focusing on the baseline abundance of *Prevotella* and ratios of *Prevotella* to *Bacteroides*. Physiological parameters were related to fermentation characteristics such as SCFA and breath H_2 excretion with the overall purpose of exploring the potential prebiotic effect. The hypothetical underlying mechanisms and metabolic effects of prebiotic DF are summarized in **Figure 2**.

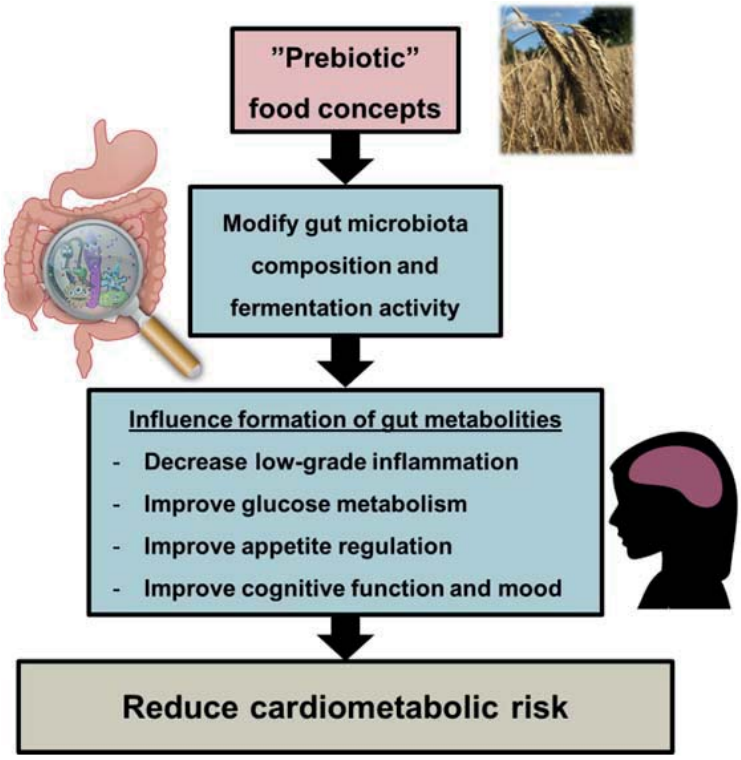

Figure 2. Flow chart including the main hypothesis regarding effect of "prebiotic" dietary fiber in the present thesis work.

Material and Methods

Test and Reference Products

Rye-based Products

In Papers I, II and III commercial blends of rye kernels (*Secale cereale* L.) were used for development and production of the test products included in the human interventions. In addition, test products developed in Papers II and III included commercially available WG rye flour and resistant starch type 2 (RS 2) from high-amylose maize (Hi-Maize® 260, 60% RS2 and 40% available starch, Ingredion Incorporated, Bridgewater, NJ, USA). The rye kernels and WG rye flour were provided by Lantmännen Cerealia (Järna, Sweden) in Papers I and II, and by Finax AB (Helsingborg, Sweden) in Paper III. Hi-Maize® 260 was provided by Kåkå (Lomma, Sweden). The rye-based products developed and included in the interventions were in the form of bread.

In Paper I, the proportion of cereal-based ingredients in the baked rye kernel bread (RKB) was 85% rye kernels and 15% white wheat flour, expressed as dry matter (dm). In Paper II the study included four different rye-based test products consisted of different dm ratios of rye kernels, WG rye flour and RS supplement. The composition of cereals and RS supplement in the breads were: **1.** 100% WG rye flour (RFB), **2.** 50% WG rye flour and 50% rye kernels (RFB/RKB), **3.** 75% WG rye flour and 25% added RS (RFB + RS), and **4.** 43% WG rye flour and 43% rye kernel with 14% added RS (RFB/RKB + RS). The last-mentioned rye-based test bread 'RFB/RKB + RS' was also included in Paper III but is abbreviated 'RB + RS'. All rye kernel breads were baked according to similar procedures; rye kernels were boiled in salted water for 30 minutes (15 minutes Paper III), followed by cooling at room temperature for 30 min. The water was completely absorbed into the kernels when cooked. The kernels were then mixed together with flour, yeast, water (and Hi-Maize, depending on if test product included RS) in a food processor (Electrolux AKM 3000, N23 N25) for 4 min (6 minutes Paper III). The dough was proofed at room temperature in a baking tin for a total of 30 minutes (2 x 15 minutes in Paper I, 20 minutes Paper III). The baking tin was covered with aluminum foil and baked at 225 °C (Paper I), 200 °C (Paper II) or 180 °C (Paper

III) for approximately 60 min (until an inner temperature of 96 °C was obtained) with a pan of water present in the oven to maximize steam. After baking, the bread was removed from the tin and wrapped in a wet towel to cool. The bread was then left overnight in a plastic bag (except Paper I where RKB was cooled for 2 hours and then directly sliced into portions). Thereafter, the bread was sliced into portions and wrapped in aluminum foil, placed in plastic bags and stored in a freezer (–20°C). The flour-based rye breads in Paper II were baked in a similar way, except for the step including boiling of kernels and instead the salt was added to the mixing bowl. The recipes and full baking procedure are accessible in Paper I, II and III.

Barley Kernel-based Product

In Paper IV, the test product consisted of commercially available barley kernels that were slightly polished (Finax, Helsingborg, Sweden). Barley kernels (85% of dm) and white wheat flour (15% of dm). The baking procedure of BKB included boiling of 595 g barley kernels (Finax) in 520 g water with 5 g added salt for 20 min and then cooled for 30 min at ambient room temperature. The water was completely absorbed into the kernels when cooked. Wheat flour (105 g), dry yeast (6 g) and water (300 g) was added to the kernels. The dough was kneaded for 4 min in a food processor (Electrolux AKM 3000, N23 N25) and was first proofed for 30 min in a bowl, followed by a second proofing (35 min) in a baking tin. The baking tin was covered with aluminum foil and baked in a household oven at 225°C with a pan of water present to maximize steam until the inner temperature of the bread reached 96°C. After baking, the bread was removed from the tin and wrapped in a wet towel to cool. The towel was removed from the bread after cooling and the bread was placed in a plastic bag at room temperature overnight. The day after, the bread was sliced into portion sizes and wrapped in aluminum foil, placed in plastic bags and stored in a freezer (–20°C).

WWB (Reference Product)

In all four papers the reference product was a low DF white wheat flour-based bread (WWB) baked according to the same recipe. In Paper I, II and IV, the WWB was baked in a bread baking machine: Severin model nr. BM 3983; Menu choice, program 2 [white bread, 1000 g, quick (time 2:35)]. The bread ingredients were 540 g white wheat flour (Kungsörnen AB, Järna, Sweden), 360 g water, 4.8 g dry yeast and 4.8 g NaCl. In Paper III, the WWB was baked in a bakery based on the same recipe and the ingredients mixed for 6 minutes, then the dough rested for 30 minutes, proofed for 35 minutes and baked at 200 °C for 40 minutes. In Papers I-

IV, the bread crust was removed after cooling to ambient temperature, then it was sliced, wrapped in aluminum foil in portion sizes, put into plastic bags and stored in freezer (-20 °C).

The bread portions were distributed in frozen state to the test subjects with instructions to put them in their freezer at home without any delay. At the day of ingestion the test subjects were instructed to thaw the bread at ambient temperature without opening the plastic bag or the alumina foil.

Servings

The portion sizes of test and reference products (Papers I-IV) were determined based on the amount of available starch Determination of available starch is further explained in sections "Chemical Analyses of Test and Reference Products". The test and reference products were either ingested as a single evening meal prior to an experimental day or during three consecutive days with the last portion consumed in the evening prior to an experimental day. The amount ingested of the evening test and reference products the day prior to an experimental day was based on 50 g available starch (Papers I-IV). The daily portion sizes provided 50 g (Paper I and Paper II), 75 g (Paper III) or 100 g (Paper IV) of available starch per day. During the first two days (Papers III and IV) it was recommended to include the bread in the daily diet and no time restrictions were set. On the third day, a portion of 25 g (Paper III) or 50 g (Paper IV), respectively, was equally divided to be ingested at breakfast and lunch. The remaining portion corresponding to 50 g of available starch was to be ingested in the evening, similar to Paper I and Paper II.

Standardized Breakfast and Lunch Meals

Standardized Breakfast

All four papers included a standardized breakfast consisted of a WWB as a glucose challenge, served with 200 ml (Papers I-III) or 250 ml (Paper IV) of tap water. The portions size of the WWB was based on 50 g available carbohydrates. The bread was baked in a home baking machine (Papers I-II and Paper IV) or at a bakery (Paper III), see above in section "WWB (reference product)").

Ad libitum Lunch

In Paper II an *ad libitum* lunch was served at the experimental day. The lunch consisted of a fried mix of diced potato, meat and onions i.e. Swedish hash (Felix Krögarpytt Klassisk, Orkla Foods Sverige AB, Eslöv, Sweden). The test subjects were allowed to choose the amount and number of servings freely. Quantities were registered (weighed) by the study leader. A glass of 100 ml of tap water was served directly prior to the lunch.

Chemical Analyses of Test and Reference Products

A summary of the composition of carbohydrates (available- and resistant starch and DF (soluble and insoluble)) in the test and reference products is presented in **Table 2** as %dm and in **Table 3** as gram per daily portion. The energy content of the *ad libitum* lunch meal (234 kcal/100 g of Swedish hash) in Paper II was based on previous analysis by Johansson et al. (2013) showing that the proportion of macronutrients (% dm) in Swedish hash was 38.5% carbohydrates, 13.9% protein and 27.5% fat [11].

Total, Available and Resistant Starch

In all four papers, the test and reference products were analyzed with respect to total starch [77] and RS [78]. The total starch was analyzed on air dried and milled samples. The RS analysis was made on products as eaten and included a pre step of chewing the product to mimic physiological conditions of grinding and mixing with alpha amylase in the saliva. The available starch in the products was calculated by subtracting RS from total starch. However, available starch in WWB (Paper III) was determined according to Holm et al (1986) [79].

Dietary Fibers

Insoluble and soluble DF content was analyzed using an enzymatic gravimetric method by precipitation of the soluble fibre with ethanol 80% (v/v) (Fibertec System E, Höganäs, Sweden) [80]. Samples of prepared products were air dried and milled prior to analysis.

Fructo-oligosaccharides and Monosaccharides

In Paper II, the fructo-oligosaccharides and inulin (henceforth referred to as fructans) content and amount of AX in the test products were analyzed in duplicates. Fructans were analyzed using the enzymatic/spectrophotometric AOAC method 999.03 and the enzyme assay kit K-FRUC (Megazyme, Bray, Ireland). The samples were treated with α-galactosidase to avoid possible interference of raffinose-series oligosaccharides [81]. The AX content was determined by using the Uppsala method, analyzing the monomeric composition in the isolated dietary fiber residue of the test products [82]. The amount of xylose, galactose and arabinose was used to calculate the AX content, and the amount was corrected for presents of arabinogalactan assuming ratio 0.69 between arabinose and galactose [83].

Meal studies

An overview of the study cohorts, experimental design and test products included in Papers I-IV are presented in **Table 4**.

Test Subjects

In Paper I (10 women and 9 men) and Paper II (11 women and 10 men) the studies included young healthy test subjects aged 25.6 ± 3.5 and 25.3 ± 3.9 years, respectively, and with BMI 21.9 ± 1.9 and 22.7 ± 2.3 kg/m^2, respectively (age and BMI expressed as mean ± SD). Paper III (30 women and 8 men) and Paper IV, (24 women and 9 men) included healthy middle-age subjects aged 63.9 ± 5.5 and 64.0 ± 5.8 years, respectively, with BMI 24.2 ± 2.5 and 24.0 ± 3.2 kg/m^2, respectively. All subjects were overall healthy, non-smokers, no known metabolic disorders and food allergies, and had normal fasting blood glucose concentrations (≤ 6.1 mmol/L). Written informed consent was received from each subject. All studies were approved by the Regional Ethical Review Board in Lund and were registered at ClinicalTrials.gov (Paper I: NCT02093481; Paper II: NCT02347293; Paper III: NCT03275948; Paper IV: NCT02427555).

Experimental Design

A randomized crossover design was applied in all studies, see **Figure 3** for a schematic overview. In Paper I the test and reference products were ingested both as a single evening meal and during three consecutive evenings at 2130 h. In Paper II the test and reference products were ingested as a single evening meal at 2100 h prior to experimental day. In Paper III and IV the test subjects ingested the test and reference product during three consecutive days, commencing the last portion at 2100 h prior to experimental day. At the experimental days fasting and postprandial measurements were obtained just prior to and following a breakfast consisting of high-GI WWB. In Papers I, III and IV the postprandial time period was 0-180 min and in Paper II the postprandial time period was 0-210 min. In Paper II, an *ad libitum* lunch was served 210 min after commencing the standardized breakfast. Initiation of breakfast is represented by time point 0 min.

In Paper IV, a selections process of test subjects occurred prior to the randomized crossover study. Thus, fecal samples were collected prior to intervention (baseline) from 99 normal- or slightly overweight human donors between 50-70 years (see "Physiological test variables" for more information regarding fecal storage and analysis). The gut microbiota composition at baseline was characterized with respect to abundance of *Prevotella* and *Bacteroides* in faecal samples from the total cohort (n = 99) From this cohort, subjects with the highest concentrations of *Prevotella* and/or *Bacteroides* and ratio of *Prevotella*/*Bacteroides* were selected (n = 33) and divided into three separate groups ('high *Prevotella* ', HP, n=12; 'low *Prevotella'*, LP, n=13; 'high *Prevotella* & high *Bacteroides'*, HPB, n=8) to participate in the randomized crossover study including the barley kernel bread (BKB) intervention.

During the study periods in Paper I and Paper III-IV, the test subjects were encouraged to standardize their meal patterns but maintain their regular eating habits and to fill in meal records. Additional instructions included avoidance of food rich in DF and alcohol during the intervention periods, and to avoid excessive physical exercise the day prior to the experimental day. In Paper II, the standardization of meal pattern and avoidance of alcohol, DF and excessive physical exercise were carried out the day prior to the experimental days. In addition, both antibiotics and probiotics should not have been taken 2 weeks (Paper I and II) or 4 weeks (Paper III) prior to the study and during the entire study. In Paper IV, no antibiotics or probiotics should have been consumed within three weeks prior to fecal donation, during the selection process and during the following dietary intervention. All studies included preparation and ingestion of test and reference products by the test subjects in their home. Hence, detailed instructions were given regarding the products to the test subjects in terms of defrosting, allowed drinks (water, coffee or tea, no milk or sugar) and scheduled

time for commencing products. After finishing the last evening meal prior to an experimental day, the test subjects should remain fasting until arriving to the research facility the next morning, at approximately 0730 am. Before the standardized breakfast was served, fasting blood samples were collected capillary and venous blood sample was drawn via a syringe (Paper III) or via an intravenous cannula (BD Venflon™ Pro Saftey Shielded IV Catheter, Becton Dickson) inserted into an antecubital vein for repeated blood sampling during the experimental day (Papers I and IV). The blood sample collection in Paper II only included capillary samplings, and in Paper III venous sampling was performed only at fasting. In addition, appetite and breath H_2 were registered at fasting. In all Papers, collection of blood samples and registration of appetite and breath H_2 were repeatedly performed after the standardized breakfast. Breakfast was served at approximately 0800 am and the test subjects finished eating within 12 minutes. The test subjects were encouraged to maintain a low physical activity during the experimental day and were recommended to perform computer work or reading.

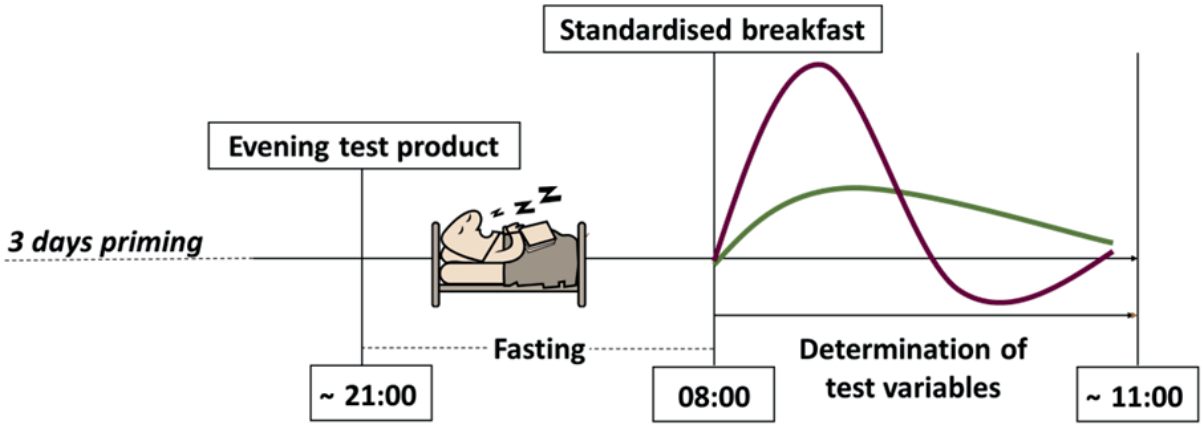

Figure 2. Schematic overview of the experimental design of meal studies included in this thesis.
A randomized crossover designs were used with some variations between studies, for example inclusion of 3 days priming (Papers I, III, IV) and/or as a single evening meal (Papers I and II) of test product.

Physiological Test Variables

Blood Glucose

In all studies, blood (b-) glucose concentrations were determined by collecting finger-prick capillary blood samples, which were analyzed using HemoCue® B-glucose equipment (HemoCue AB, Ängelholm, Sweden).

Serum Insulin

In Papers I and IV venous blood sampling was used for serum (s-) insulin determination. In Papers II and III capillary blood samples were used for s-insulin determination. All samples were analyzed using a solid phase two-site enzyme immunoassay kit (Insulin ELISA 10-1113-01, Mercordia AB, Uppsala, Sweden).

Serum FFA and Triglycerides

FFA concentrations in serum from venous (Papers I, III and IV) or capillary (Paper II) blood sampling were determined using an enzymatic colorimetric method (NEFA, ACS-ACOD method, WAKO Chemicals GMbH, Neuss, Germany). In Papers I and III, s-triglycerides were determined with a multi-sample enzymatic assay (LabAssay™ Triglyceride 290-63701, GPO DAOS method, Wako Chemicals GMbh, Neuss, Germany).

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

CRP concentrations in serum (Paper I) and in plasma (Papers III and IV) were determined with an enzyme immunoassay (CRP ELISA kit, Immunodiagnostik AG, Bensheim, Germany). PAI-1 concentrations in serum (Paper I) and IL-6 concentrations (Papers I-IV) were obtained using an enzyme immunoassay (Human PAI-1 ELISA, RBMS2033R, Bio Vendor GmbH, Heidelberg, Germany and Human IL-6 HS600B, R&D Systems, Abingdon, UK, respectively). Venous blood samples were collected for IL-6 analysis in Paper I (serum), and in Paper III and IV (plasma). In Paper II, IL-6 was analyzed in capillary serum samples. Enzyme immunoassay was also used to determine IL-18 (Human IL-18 ELISA Kit 7620, MBL Medical & Biological Laboratories CO., Ltd, Nagoya, Japan) in serum (Paper I) and plasma (Paper III), respectively. Modification of IL-18 analysis procedure was made by not diluting of IL-18 samples prior to analysis. In Paper III, plasma concentrations of IL-1β and LBP were determined with an enzyme immunoassay (Human IL-1β HSLB00D, R&D Systems, Abingdon, UK and Human LBP ELISA Kit EKH3120, Nordic Biosite, Täby Sweden, respectively).

Plasma Ghrelin, GLP-1, GLP-2 and PYY

Ghrelin (Paper I), GLP-1, GLP-2 and PYY (Papers I, III and IV) were analyzed in venous blood samples. PYY concentration in Paper II was determined in capillary blood samples. Blood samples intended for analysis of Ghrelin, GLP-1, GLP-2 and PYY in plasma were collected in tubes with added DPPIV inhibitor, 10 µl/ml blood (Millipore, St Charles, MO, USA) and aprotinin, 50 µl/ml blood (Sigma Aldrich, St Louis, MO, USA). In addition, aliquoted plasma intended for ghrelin was treated with 1 M HCl (10:1) prior to freezing. An enzyme immunometric assay was used to determine p-ghrelin (Human Acylated Ghrelin ELISA RD194062400R, Bio Vendor GmbH, Heidelberg, Germany). GLP-1 in plasma

was analyzed using highly sensitive ELISA kit (GLP-1 (Active 7-36) ELISA 43-GP1HU-EO1 ALPCO Diagnostics, Salem, NH, USA). Competitive enzyme immunoassay was used to determine GLP-2 (Human GLP-2 EIA YK141, Yanaihara Institute Inc. Shizuoka, Japan) and PYY, both PYY (3-36) and PYY (1-36) (Human PYY EIA YK080, Yanaihara Institute Inc. Shizuoka, Japan).

Adiponectin

Adiponectin in serum was analyzed in Paper I with a solid phase two-site enzyme immunoassay kit (Adiponectin ELISA 10-1193-01, Mercordia AB, Uppsala, Sweden).

Nesfatin-1

In Paper I, p-nesfatin-1 was determined using a sandwich enzyme immunoassay (Human Nesfatin-1 ELISA RD191227200R, Bio Vendor GmbH, Heidelberg, Germany).

BDNF

In Paper III, enzyme immunoassay was used to determine BDNF in plasma (ChemiKine BDNF Sandwich ELISA kit CYT306, Millipore Bioscience Research Reagents, USA and Canada).

SCFA

The SCFA (acetate, propionate and butyrate) in plasma were analyzed in Papers I and IV using a gas-chromatography method with 2-ethyl butyrate as an internal standard [84].

Breath H_2

Breath hydrogen expiration was used as an indicator of colonic fermentation in all four studies and was measured using an EC 60 gastrolyzer (Bedfont EC60 Gastrolyzer, Rochester, England).

Subjective appetite ratings

Subjective appetite ratings (satiety, hunger and desire to eat) were included in all studies using a 100 mm Visual Analog Scale (VAS) graded from “not at all” to “extreme” [85]. In Papers I and III, IV, the VAS was provided on paper sheets to mark with a pen. The VAS in Paper II was computer-based using the mouse cursor to rate the appetite on the digital 100 mm scale.

Mood

In Paper III, the Swedish Core Affect Scale (SCAS) was used to assess the mood measurements. SCAS includes three self-reporting rating scales for evaluating valence and for activation, respectively [86], and the ratings of subjective mood were registered using 100 mm VAS. The rating of valence was measured by obtaining a mean rating of the three scales graded from depressed-happy, displeased-pleased and sad-glad, respectively. The activation rating was evaluated by obtaining a mean rating of the three scales graded from dull-peppy, sleepy-awake and passive-active, respectively [86].

Working Memory

In Paper III, the working memory (WM) tests were used to evaluate the ability to simultaneous process and store of information. The tests are based on a work by Daneman and Carpenter [87] but have been modified according to Radeborg et al [88]. Each WM test session consisted of 48 short, declarative sentences, where half of the sentences in each session were semantically meaningful e.g. 'The father got a present' and the other half was nonsensical e.g. 'The brother was grandmother'. The sentences were divided into 12 sets with 3, 4 or 5 sentences in each set (i.e. four of each set per session), and the subjects were blinded to number of sentences per set. All WM testing sessions were conducted by the same experimenter that read the sentences to the subject at a rate that was slow and steady. The sentences were designed so that the subjects were required to listen to the complete sentence before they could judge its meaning. The subjects were instructed to answer directly after each sentence was read and state if the sentence was 'correct' (i.e. semantically meaningful) or 'incorrect' (i.e. nonsensical). The test subjects knew that a set was finished when the experimenter said 'Now', which indicated that the subject *now* should attempt to repeat the first noun in each sentence (in any order). The variable presented as results are the total number of first nouns remembered. WM tests were performed three times during the experimental day (at 90,120 and 160 min) after each intervention periods, i.e. six times in total. Each test took approximately 8 min to complete. The order of the WM test was the same for all subjects but half of the group started with reference product (n=19) and the other half started with the test product (n=19).

Selective Attention Test

In Paper III, a computerized selective attention (SA) test was used to, measure the ability to sustain a prolonged attention, and to control and split the attention to the entire picture shown on the computer screen. The SA test requires storing and processing information simultaneously, analogous with the WM tests. However, the time to store information is shorter but the pressure of time is greater. The SA test included 96 pictures and each picture was shown on the screen for 2 s. The SA

test was performed according to Nilsson et al [89], and one SA test took approximately 10 min to complete. The SA test was performed twice per experimental day at 30 and 170 min post the standardized breakfast, i.e. four SA tests in total. The SA test results were based on the number of correct responses (total 95 credits) and reaction time to give an answer, i.e. press a key on the keyboard.

Fecal Microbiota

Subjects were provided labelled sterile tubes (Sarstedt, Nümbrecht, Germany) and collected fecal sample themselves. The samples were immediately frozen and handed over to the experimental department within 24 h for continued storage at -80°C until analysis. In Paper IV, genomic DNA purification of the fecal samples and 16S rRNA quantitative PCR analysis were performed in collaboration with Wallenberg Laboratory, Department of Molecular and Clinical Medicine, University of Gothenburg, Gothenburg, Sweden. More detailed information is included in the method section in Paper IV.

Table 2.
Composition of available and indigestible carbohydrates in the test and reference products presented as % dry matter[1]

(Paper no.) Products	Starch			NSP		Total DF	Fructans[2]	AX[2]
	Total starch	Available starch[3]	RS	Insoluble DF	Soluble DF			
Composition				*% dry matter*				
(I-IV) WWB	77.7±1.6	76.1±1.4	1.9±0.1	3.4±0.3	1.5±0.3	6.4±0.6	0.5	0.5
(I) RKB	66.6	61.8	4.8	12.3	3.8	20.9	6.1	4.0
(II) RFB	59.3	56.0	3.4	13.9	5.1	22.4	3.3	7.3
(II) RFB/RKB	58.2	51.2	6.9	14.6	4.4	25.9	3.9	7.0
(II) RFB+RS	67.4	54.2	13.2	15.9	3.5	32.6	2.5	5.0
(II) RFB/RKB+RS	71.5	58.9	12.7	14.3	4.1	31.1	3.2	6.3
(III) RB+RS	63.0	52.9	10.1	14.8	4.0	28.9	-	-
(IV) BKB	65.5	57.2	8.4	8.4	4.0	20.8	-	-

[1]Values of available starch, fructans and AX are based on means of 2 replicates, RS means of 6 replicates, NSP means of 3 replicates. [2]Analysis of fructans and AX was only performed on RKB in Paper I and on all products in Paper II. [3]Available starch was obtained by calculating the difference between total starch and RS, except for WWB in Paper III that was analyzed with respect to available starch according to Holm *et al* (1986) [79]. Total DF include RS, and insoluble and soluble NSP. Analyzing methods are presented in section *Chemical Analyses of Test and Reference Products*. (-) indicates that no analysis has been performed or data not available. RKB, rye kernel-based bread; RFB, rye flour-based bread; RFB/RKB, rye flour and kernel-based bread (1:1 ratio); RFB+RS, RFB with added RS (25% dm); RFB/RKB+RS, RFB/RKB with addition of RS (14% dm); RB+RS, rye flour and kernel-based bread (1:1 ratio) with added RS (14% dm); BKB, barley kernel-based bread; WWB, white wheat flour-based reference bread; DF, dietary fiber; NSP, non-starch polysaccharides; RS, resistant starch; AX, arabinoxylans.

Table 3.

Composition of available and indigestible carbohydrates in the test and reference products displayed as gram per daily portion[1]

(Paper no.) Products	Starch			NSP		Total DF	Fructans[2]	AX[2]
	Total starch	Available starch[3]	RS	Insoluble DF	Soluble DF			
Portion size				*g / daily portion*				
(I-II) WWB	51.2±51.2	50.0±0.0	1.2±0.1	1.8±0.3	0.8±0.3	3.8±0.2	0.3	0.3
(III) WWB	75.6	75.0	-	3.9	1.2	5.1		
(IV) WWB	103	100	2.7	5.3	2.7	10.7		
(I) RKB	53.9	50.0	3.9	9.0	2.8	15.7	5.0	3.2
(II) RFB	53.0	50.0	3.0	11.0	4.1	18.1	3.0	6.5
(II) RFB/RKB	56.8	50.0	6.8	12.5	3.7	23.0	3.8	6.8
(II) RFB+RS	62.1	50.0	12.1	11.9	2.9	26.9	2.3	4.6
(II) RFB/RKB+RS	60.8	50.0	10.8	9.9	3.1	23.8	2.7	5.3
(III) RB+RS	89.3	75.0	14.3	20.9	5.7	40.9		
(IV) BKB	115	100	14.7	14.7	7.0	36.4		

[1]Values of available starch, fructans and AX are based on means of 2 replicates, RS means of 6 replicates, NSP means of 3 replicates. [2]Analysis of fructans and AX was only performed on RKB in Paper I and on all products in Paper II. [3]Available starch was obtained by calculating the difference between total starch and RS, except for WWB in Paper III that was analyzed with respect to available starch according to Holm *et al* (1986) [79]. Total DF include RS, and insoluble and soluble NSP. Analyzing methods are presented in section *Chemical Analyses of Test and Reference Products*. (-) indicates that no analysis has been performed or data not available. RKB, rye kernel-based bread; RFB, rye flour-based bread; RFB/RKB, rye flour and kernel-based bread (1:1 ratio); RFB+RS, RFB with added RS (25% dm); RFB/RKB+RS, RFB/RKB with addition of RS (14% dm); RB+RS, rye flour and kernel-based bread (1:1 ratio) with added RS (14% dm); BKB, barley kernel-based bread; WWB, white wheat flour-based reference bread; DF, dietary fiber; NSP, non-starch polysaccharides; RS, resistant starch; AX, arabinoxylans.

Table 4.
Overview of studies, subjects, test products, interventions and test vaiables included in the thesis work[1]

Paper	Subjects (Age / BMI)	Test products	Intervention	Test variables
I	n = 19 25.6 ± 3.5 years 21.9 ±1.9 kg/m^2	Rye kernel bread (**RKB**) WWB	Single evening meal & three days consecutive evening meal	b-glucose, s-insulin, p-GLP-1, p-GLP-2, p-ghrelin, p-PYY, p-nesfatin, s-FFA, s-CRP, s-IL-6, s-IL-18, s-adiponectin, s-PAI, s-TG Breath H_2, p-SCFA Subjective appetite sensations
II	n = 21 25.3 ± 3.9 years 22.7 ± 2.3 kg/m^2	Rye flour bread (**RFB**) Rye flour- and rye kernel bread (**RFB/RKB**) Supplementation with RS2 (**+RS**) WWB	Evening meal	b-glucose, s-insulin, p-PYY, s-FFA, s-IL-6 Breath H_2 Subjective appetite sensations Voluntary energy intake
III	n = 38 63.9 ± 5.5 years 24.2 ± 2.5 kg/m^2	Rye flour- and kernel bread with RS2 supplementation (**RB+RS**) WWB	Three days priming incl. evening meal	b-glucose, s-insulin, p-GLP-1, p-GLP-2, p-PYY, s-FFA, p-CRP, p-IL1β, p-IL-6, p-IL-18, p-LPB, s-TG, p-BDNF Breath H2, p-SCFA Subjective appetite sensations, mood ratings, working memory test, selective attention test
IV	n = 33 64.0 ± 5.8 years 24.0 ± 3.2 kg/m^2	Barley kernel bread (**BKB**) WWB	Three days priming incl. evening meal	b-glucose, s-insulin, p-GLP-1, p-GLP-2, p-PYY, p-nesfatin, s-FFA, s-CRP, s-IL-6, s-IL-18, s-adiponectin, s-PAI, s-TG Breath H2, p-SCFA Subjective appetite sensations

[1]Values are presented as means ± SD. WWB, white wheat bread (reference); RS, resistant starch.

Calculations and Statistical Methods

Data are presented as means ± standard error of the mean (SEM) and values of $P < 0.05$ are considered significant. Test subjects served as their own controls in Papers I-IV. In addition, in Paper IV results were also compared between different subject groups based on baseline gut microbiota composition. Glucose and insulin are presented as incremental areas under the curve (iAUC), using fasting value as baseline. However, if the fasting values of glucose and insulin differed significantly; the data are also presented as area under the curve (AUC) using absolute values (Paper IV). All remaining test variables are presented as means and/or AUC. The calculations of iAUC and AUC are based on the response of each individual test subject and meal using the trapezoid model. Graph plotting and calculation of AUC were made using GraphPad Prism (version 6, GraphPad Software, San Diego, CA, USA). The individual concentration maximums in test variables for each subject and test product were used for determination of Peak- and/or incremental Peak (iPeak) values of glucose and insulin. In Paper I, III and IV, the insulin sensitivity was determined by using the composite insulin sensitivity index ($ISI_{composite}$). However, the method was modified as the breakfast (WWB) consisted of 50 g available carbohydrates instead of 75 g of glucose [90]. $ISI_{composite}$ was calculated by using the formula: 10 000/square root of [fasting glucose (mg/dl) × fasting insulin (μU/ml) × mean glucose concentrations 0-120 min (mg·min/dl) × mean insulin concentrations 0-120 min (μU·min/ml)]. The mean glucose and insulin concentrations included in the calculations were based on blood glucose and s-insulin values measured every 30 minutes in the postprandial phase post the standardized breakfast. In Papers III and IV, homeostatic model assessment of insulin resistance (HOMA-IR) was calculated as: [fasting blood glucose (mmol/L) × fasting insulin (mU/L) / 22.5] [91].

Evaluation of significant differences in test variables in Papers I-IV were performed with ANOVA (General Linear Model) using the statistical software MINITAB (release 17; Minitab, Minitab Inc, State College, PA, USA). In Paper I, the data was analyzed as a 2x2 factorial design. The independent main variables were the test products (RKB and WWB) and length of priming (1 day and 3 days), additionally, interactions between these independent variables were examined. In Paper IV, the data was analyzed as a 2x3 factorial design including evening meals (BKB and WWB), i.e. 'Meal', and baseline gut microbiota composition (HP, LP and HPB), i.e. 'Microbiota', as independent main variables. In Papers I and IV, the ANOVA was then followed by the post hoc analysis: Tukey's pairwise multiple comparison method (MINITAB Statistical Software). In Paper II, Dunnett's test was used to compare the rye-based test products with the WWB reference bread.

Furthermore, in Paper IV, a second approach was applied only including the groups HP and LP, excluding the HPB group. Consequently, the 'Microbiota' variable in the 2x2 factorial design included only the "extreme" groups with respect to ratios of *Prevotella*/*Bacteroides* at baseline. In addition, the metabolic responses depending on treatment in Paper IV were evaluated separately in each microbiota subgroup using one-way ANOVA analysis (MINITAB Statistical Software). In the case of unevenly distributed residuals (tested using Anderson-Darling normality test and considered unevenly distributed if $P < 0.05$), transformation using Box Cox was performed on the data prior to the ANOVA analysis. Test variables with very little change over time are presented as weighted mean, including an overall mean based on a mean per hour. If a test value for one test variable is missing for a subject, that subject will not be included in the particular statistical evaluation of the test variable. In Papers I, II and III, the differences between the interventions at different time points during the experimental day ('Time') were evaluated using a mixed model (PROC MIXED in SAS release 9.3; SAS Institute Inc, Cary, NC) with repeated measures and an autoregressive covariance structure for the test variables included in the study. In addition, Pearson correlation was used to investigate the relationships between selected test variables (Minitab Statistical Software). Primary outcome measure for power calculations was change in blood glucose iAUC (0–120 min) (Papers I, II and IV) or changes in cognitive performance (Paper III).

Results and Discussion

Paper I

Rye-based evening meals favorably affected glucose regulation and appetite variables at the following breakfast; a randomized controlled study in healthy subjects

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The purpose of Paper I was to investigate possible beneficial effects of rye kernel-based food. For this purpose, healthy test subjects consumed a rye kernel bread (RKB), rich in DF, in the evening and cardiometabolic risk factors and appetite regulation were determined at fasting and postprandial phase following a standardized breakfast consumed the next morning, i.e. on the experimental day. The results on test variables after the RKB were compared to results after a white wheat bread (WWB, reference). The test and reference evening meals were based on 50 g available carbohydrates. Two different priming settings (a single evening meal "1d" or three consecutive evening meals "3d") were applied prior to the experimental day to investigate the efficacy of DF in WG rye products to improve the metabolic risk markers in the overnight study model. Test variables obtained in the morning, i.e. 10.5–13.5 h after intake of the test or reference evening meal, included measures of glucose regulation, inflammatory tone, appetite regulation (gut hormones and nesfatin-1), markers of colonic fermentation activity and perceived appetite sensations. The study design was a randomized cross-over design and included nineteen healthy young adults with normal BMI.

In the present study, the results disclosed that the effects on test variables of the RKB were observed independently of length of priming (1d vs 3d, $P > 0.05$), with the exception of serum CRP and a tendency for plasma propionate. Therefore, the results are presented as 'WWB' and 'RKB', including both 1d and 3d data for respective test product.

Results and Discussion

Intake of RKB evening meal decreased both the incremental glucose and insulin areas under the curve (iAUC 0-120 min) by 23% ($P = 0.001$) and 13 % ($P < 0.05$), respectively. In addition, the postprandial incremental peak of b-glucose was significantly lower following the standardized breakfast after the RKB evening, compared to the WWB evening meal (iPeak, -16%, $P < 0.01$). The applied overnight study design has not previously been performed with RKB products. However, the study design has been applied using barley kernel products as evening meals [11, 44], displaying beneficial effects on postprandial glucose and insulin response in accordance with present findings after RKB. Nevertheless, in a previous study, rye kernel-based food ingested at breakfast beneficially affected day-long glucose homeostasis in healthy subjects [33], i.e. the cumulative glucose iAUC response (0-120 min) after breakfast, lunch, and dinner (up to 11.5h after breakfast). In the present paper, the RKB evening meal resulted in significantly lower serum concentration of FFA at fasting (-14%, $P < 0.05$), i.e. 10.5 h after intake, compared to the WWB evening meal. Maintaining low concentrations of FFA is important from a metabolic standpoint and, consequently, an elevation of FFA concentrations in healthy non-diabetic subjects via lipid infusion to simulate FFA concentrations comparable with subjects with obesity and T2D resulted in significantly decreased insulin sensitivity [92]. Thus, it is possible that the significantly lower FFA concentrations after RKB may have contributed to the improvement seen in glucose regulation.

A significant increase of GLP-1- and PYY concentrations was noticed at fasting (23%, $P < 0.05$ and 6%, $P < 0.01$, respectively) and during the breakfast postprandial phase (12%, $P < 0.01$ and 9%, $P < 0.001$, respectively) after RKB evening meals compared to WWB. The gut hormones PYY and GLP-1 are involved in both appetite and glucose regulation. Thus, it is plausible to suggest that the increase may have contributed to the improved glucose tolerance detected after the RKB. Furthermore, nesfatin-1 has shown involvement in glucose control and reduction in food intake [93]. No significant differences in nesfatin-1 concentrations depending on evening meal were seen in the present work, however a trend towards increased nesfatin-1 concentrations was observed at fasting after RKB (3%, $P = 0.095$). In addition, a trend towards lowering the gastrointestinal hunger-inducing hormone ghrelin was observed during the whole experimental day (AUC 0-180 min, -7%, $P = 0.086$) after the RKB evening meal in comparison to WWB.

In accordance with increased concentrations of satiety-related hormones and a decrease in hunger-related hormone concentrations, subjective appetite sensations were improved after the RKB evening meal. Accordingly, the intake of the RKB evening meal resulted in increased satiety (AUC 0-180 min, 14%, $P < 0.05$) at the

subsequent breakfast, as well as a significant decrease in the subjective appetite ratings of hunger and desire to eat (both: AUC 0-180 min, -9%, $P < 0.05$). This is, to the best of our knowledge, the first study to observe effects of rye kernel-based food on subjective appetite sensations in this time perspective. However, rye kernel-based meals served as breakfast have previously been shown to induce increased feelings of satiety in the postprandial phase directly after intake, and also in the afternoon, 4 h post a second meal [12, 94].

No significant differences in insulin sensitivity, GLP-2 concentrations or concentrations of inflammatory markers (CRP, IL-6, IL-18), adiponectin, PAI-1 or triglycerides ($P > 0.05$) were noted depending on intervention.

Rye is a rich source of DF, and a high proportion of the DF is highly fermentable [42]. According to the literature, rye consists of approximately 8.5-11.5% AX, thus constituting one of the major DF components in this cereal [95]. Rye has also a high concentration of fructans (around 4%) compared to other cereals (wheat, oat and barley). [42]. The DF composition regarding soluble and insoluble DF, and RS in the RKB and WWB (reference) is published data [96] and presented in Table 2 and Table 3. In addition, the AX and fructan content in the RKB was determined using the Uppsala method [82] and enzymatic/spectrophotometric AOAC method 999.03. RKB was 6.1% dm AX (5.0 g AX per daily test portion), and the fructan content was 4.0 % dm (3.2 g per test portion) (unpublished data).

RKB increased concentrations of total and individual SCFA (acetate, propionate and butyrate) in plasma at fasting, and induced higher concentrations of breath hydrogen excretion compared to the WWB reference, indicative of increased gut fermentation activity promoted by the DF in RKB. The results are in accordance with previous observations showing increased SCFA concentrations in pigs consuming a diet rich in NSP, including AX and fructans (from rye flakes and enzyme-treated wheat bran), compared to a low-DF western diet [97]. SCFA has been shown to stimulate secretion of the gut hormones PYY and GLP-1 from L-cells in several in vitro studies [63]. Interestingly, in Paper I, mean concentrations of GLP-1 were positively correlated with total SCFA after the RKB evening meal.

As previously mentioned, the results in Paper I disclosed that the beneficial metabolic effects of the RKB were observed, with few exceptions, independently of length of priming, i.e. 1 day vs 3 days. The exceptions were CRP concentrations, which decreased independently of intervention product after three days priming with RKB or WWB compared with consumption of the products as single evening meals ($P<0.05$), and a trend towards increased plasma propionate concentrations after three days priming with RKB compared to consumption of RKB as a single evening meal ($P = 0.051$). Thus, it cannot be ruled out that some of the physiological variables would have been further affected with an intervention period of more than three days.

The increase in breath hydrogen excretion and plasma concentrations of SCFA indicate a rapid increase in intestinal microbiota metabolism. Analysis of fecal microbiota was performed with the purpose of investigating possible changes in the microbiota composition as a response to RKB, as soon as after a single meal or after three days of intervention (unpublished data). The fecal samples were collected during the experimental day (the day after the last test portion of RKB or WWB) and were analyzed using polymerase chain reaction (PCR) technique. The results showed that the microbiota composition was significantly altered at the family level after RKB compared to WWB, independently of priming setting i.e. changes in microbiota composition had already occurred the day after a single evening meal of RKB. Thus, RKB significantly increased the abundance of Prevotellaceae and Ruminococcaceae, and decreased the abundance of Bacteroidaceae ($P < 0.05$), compared to WWB. In addition, the pooled data at genus level showed that RKB significantly increased the abundance of *Prevotella* ($P < 0.01$) and *Fecalibacterium* compared to WWB ($P < 0.05$), and tended to lower the abundance of *Bacteroides*. ($P = 0.06$), see **Figure 4**.

Effects of diet on gut microbiota composition have previously been studied mainly with respect to the influence of long-term diet. Only a few studies have investigated the effects of short-term diet interventions. However, the results of the present study are in accordance with a previous study observing increased ratio of gut *Prevotella*/*Bacteroides* after a 3-day intervention with barley kernel-based bread [13]. Moreover, a short-term intervention with an animal-based diet with nearly no DF intake altered the β-diversity after 2 days of intake [98]. and further, an increased postprandial endotoxemia (based on LPS concentrations) was noticed in response to a single high-fat meal [99]. Taken together, the results thus, indicate that the gut microbiota quickly may respond to changes in diet.

In conclusion, the results in Paper I show that rye kernel-based products have the potential to lower glucose and insulin responses to a standardized breakfast in healthy subjects 10.5–12.5 hours after intake. In addition, intake of rye kernel-based evening meals increased the satiety-promoting gut hormones GLP-1 and PYY the next day. The increase in these incretins was accompanied by higher subjective feeling of satiety, lower feelings of hunger and less desire to eat. The beneficial metabolic effects of rye kernel-based evening meals were associated with increased gut fermentation activity, indicated by increased plasma SCFA concentrations and breath hydrogen excretion the next day. Mean concentrations of GLP-1 were positively correlated with total SCFA after the RKB. The results from Paper I thus make it plausible to suggest that possible underlying mechanisms relating to increased gut hormone concentrations and improved glucose and appetite regulation are mediated through increased concentrations of SCFA resulting from gut fermentation. The quick shift observed in gut microbiota composition (unpublished data) after the RKB may possibly be involved.

Taken together, the results in Paper I indicate anti-diabetic and anti-obesogenic potential of rye kernel-based products.

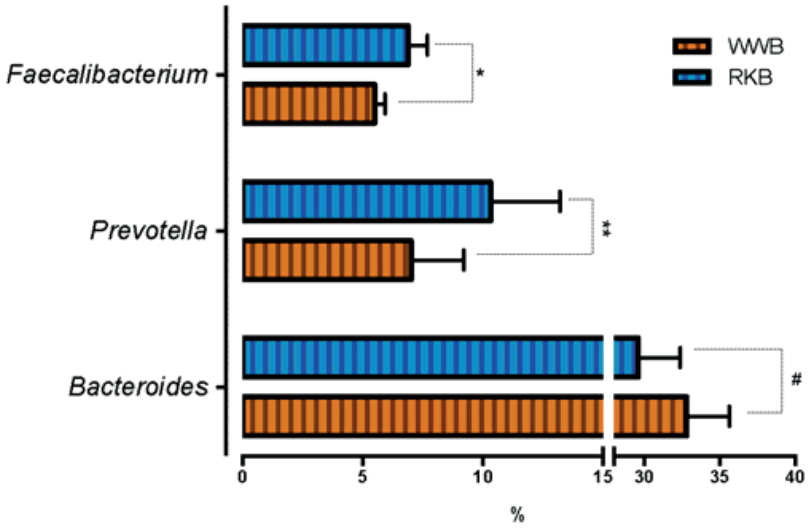

Figure 4. Differences bacterial genera between pooled results of RKB (mean 1d and 3d) and WWB (mean 1d and 3d). * P < 0.05, ** P < 0.01, # P = 0.06.

Paper II

Effects of whole grain rye, with and without resistant starch type 2 supplementation, on glucose tolerance, gut hormones, inflammation and appetite regulation in an 11–14.5 hour perspective; a randomized controlled study in healthy subjects

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Based on the beneficial metabolic responses up to 13.5 hours after intake of the rye kernel-based product in Paper I, it was relevant to study metabolic properties of WG rye flour products, and to evaluate possible differences with respect to WG rye kernels. Due to some of the RS portion of DF being lost in the process of milling kernels to flour, the intention of this Paper was also to evaluate whether supplementation of WG rye flour with commercial RS would enhance possible beneficial effects. Therefore, in Paper II, WG rye-based products, varying in structure and amount of resistant starch, were investigated with respect to semi-acute effects on cardiometabolic risk markers and appetite regulation, i.e. effects 11–14.5 hours after intake.

The study included four WG rye breads with different flour to kernel ratios and with or without supplementation with commercial RS, i.e. RS type 2 from high-amylose maize to elevate the importance of RS content. The following rye-based test products were included: 1. WG rye flour bread (RFB); 2. WG rye flour bread supplemented with 25 % RS2 (RFB + RS); 3. a blend of rye kernels and WG rye flour bread in a ratio of 1:1 (RFB/RKB); 4. WG rye bread with 1:1 ratio of rye kernels and WG rye flour, supplemented with 14 % RS2 (RFB/RKB + RS). A WWB was included as a reference product. The portions of the test and reference products were based on 50 g available carbohydrates and were provided to healthy young adults in a randomized crossover study design. Based on the results in Paper I showing no major differences when the test product was consumed as a single evening meal compared to being consumed for three consecutive evenings, the test and reference products in Paper II were provided as late single evening meals (at 2100 h). The test variables in blood (glucose, insulin, PYY, FFA and IL-6), markers of gut fermentation (breath hydrogen excretion) and subjective appetite ratings (satiety, hunger and desire to eat) were determined the next morning at fasting and repeatedly during 3.5 hours following a standardized breakfast. In addition, the energy intake was evaluated 3.5 hours after the standardized breakfast by registered intake of an *ad libitum* lunch.

Results and Discussion

In Paper II, the evening test bread RFB/RKB+RS resulted in decreased postprandial glucose and insulin responses (iAUC) by 27% and 21% ($P < 0.05$), and increased gut hormone PYY in plasma both at fasting (+17%, $P < 0.05$) and during the experimental day (mean 0-120 min, +15%, $P = 0.01$) the following morning after the standardized breakfast, compared to WWB. No such effects were seen after intake of the RFB/RKB without RS2 supplementation, or after intake of the other test products. The lack of effects of the RFB/RKB on glucose regulation and PYY indicates an importance of RS to elicit effects on these metabolic variables. On the other hand, no significant effects were observed after the RFB+RS, even if the total amounts of RS were similar to, or even slightly higher than in the RFB/RKB+RS2. The results imply that other factors than the total amounts of RS must be involved with regard to the discrepancy in effects on glucose regulation and PYY, e.g. the structure of the cereals and/or type of RS may also be important. Notably, the hypothesis of enhancing the effects of WG rye flour products on glucose and insulin response by supplementation with RS could only be confirmed when adding a relatively moderate amount of RS2 (14%) to the RFB/RKB product but not when supplementing higher concentrations, i.e. adding 25% RS2 to the RFB. Previously, differences in glucose response have been observed depending on whether the test product (WWB) was supplemented with a

combination of RS2 (similar to the RS used in the present study) and NSP (from barley), or whether these components were supplemented separately [33, 34]. Thus, a combined addition of RS and NSP to a WWB consumed in the evening displayed an improved glucose tolerance the following morning, while separate addition of either of these components to a WWB did not display the same beneficial effects on the glucose response.

While only RFB/RKB+RS significantly improved the glucose regulation, all rye products decreased fasting FFA concentrations by 17% ($P < 0.05$, trend RFB/RKB: $P = 0.057$), and thus the FFA results are in accordance with previous study including rye kernel-based bread [96]. A lowering effect on FFA concentrations may improve insulin sensitivity; however, the lack of differences in effects on FFA between the test products points towards additional underlying mechanisms possibly being involved in the discrepancy of effects on glucose tolerance. Furthermore, all rye products increased colonic fermentation activity 11–14.5 hours after intake compared to intake of WWB as determined by increased breath hydrogen excretion ($P < 0.001$). No significant effects were noted on fasting or mean concentrations of IL-6 concentrations ($P > 0.05$) depending on evening meal.

No major differences in DF composition were observed between the test products supplemented with RS (RFB+RS and RFB/RKB+RS). Instead, more pronounced differences were observed in DF composition between products with *or* without RS supplementation, i.e. RFB versus RFB+RS and RFB/RKB versus RFB/RKB+RS, in addition to the expected difference in amount of RS. The commercial RS2 (Hi-Maize 260) supplemented to the rye-based products consist of 60% RS2 and 40% available starch. Thus, as not only RS2 was added to the product, but also digestible starch, it is possible that addition of RS2 might actually dilute the "DF effects" in the supplemented rye-based products, which possibly may have occurred when adding relatively high proportions of RS2 to the RFB. In fact, consulting the chemical analysis of the test products, it can be noted that the RS supplemented rye-based products have a lower concentration of soluble DF, and decreased concentrations of fructans and AX, compared to the corresponding un-supplemented products. The effect of dilution of the non-starch DF might be a possible limitation when supplementing RS to WG products, so consequently there is a need in future research to address this potential restraint. However, the hypothesized reduced metabolic beneficial effects due to dilution of DF with available starch are probably not seen in refined cereal food products, such as white wheat flour products, in which the amounts of DF are low.

In Paper II, as reported above, intake of the RFB/RKB+RS resulted in increased PYY concentrations 11-13 hours compared to the WWB. An increase in PYY concentrations has been shown to reduce voluntary food intake, which is in

accordance with its role as a satiety-inducing gut hormone [100]. However, no significant effects were observed in *ad libitum* lunch intake or subjective appetite ratings after intake of RFB/RKB+RS. It has previously been shown that the PYY response does not always correlate with the subjective appetite ratings [101]. Instead the RFB displayed the most pronounced effects on subjective appetite ratings by increasing AUC satiety by 21% and decreasing AUC desire to eat by 20% in the 11–14.5 hours perspective after intake ($P < 0.01$), compared to WWB. In addition, both RFB and RFB/RKB decreased the AUC hunger by 15% and 17%, respectively (0-210 min, $P < 0.05$). It can be suggested that the improved appetite variables after the test products without RS supplementation are due to the higher content of AX and fructans, or the total amounts of soluble DF.

The *ad libitum* lunch in Paper II was provided 210 min after the standardized breakfast. No significant differences were observed depending on intervention. However, a slight non-significant decrease was seen in energy intake after the RFB/RKB evening meal (-7%, $P > 0.05$). It has previously been shown that an evening meal including rye kernels decreased the energy intake by a significant 7% at an *ad libitum* lunch ($P < 0.05$) [102], thus indicating a weight-regulating potential of WG rye kernel and flour-based products. One possible limitation when measuring the energy intake in the present study was the use of a low-calorie standardized breakfast, consisting of WWB and water, which was selected primarily to evaluate the postprandial metabolic responses. Obviously, such a breakfast is different from a normal habitual breakfast. Consequently, previous studies observing significant differences in *ad libitum* energy intake at lunch after rye or barley evening meals have used more realistic breakfasts in terms of complexity and calories [11, 102]. Thus, in future studies it would be interesting to include a more realistic breakfast to investigate *ad libitum* energy intake at lunch.

In conclusion, in addition to rye kernel foods, as shown in Paper I, the results in Paper II show that inclusion of WG rye flour food products also have the potential to elicit beneficial effects on glucose regulation, gut hormone, and appetite sensations in young healthy adults. Supplementation with commercial RS (high-amylose maize RS type 2) may or may not (due to dilution of DF contents) add to beneficial metabolic effects. Thus, moderate RS supplementation (14%) to a rye bread made from WG rye flour and rye kernels, at a 1:1 ratio between flour and kernels (RFB/RKB+RS), lowered glucose and insulin response at a following standardized breakfast. In contrast, higher amounts of RS (25%) added to a flour-based rye bread (RFB+RS) did not elicit any beneficial impact on the glucose tolerance, possibly due to substantial amounts of available starch in the commercial RS fraction. Intake of RFB/RKB+RS also promoted the most pronounced increase in the gut hormone PYY. However, the rye-based products not supplemented with RS, but containing the highest levels of non-starch DF, especially soluble DF (RFB and RFB/RKB), instead showed the best potential to

positively affect subjective appetite variables. The results may thus suggest that DF might differ in metabolic effects depending on types of DF, e.g. starch vs. non-starch DF, and/or soluble vs non-soluble DF.

All rye-based evening meal products that were included in Paper II decreased FFA the next morning, and increased the gut fermentation activity, using breath H2 excretion as a rough indicator of fermentation. The apparent metabolic effects of the rye products are possibly mediated via the gut fermentation of DF.

Paper III

Impact of rye-based evening meals on cardiometabolic risk factors, cognitive functions and mood in healthy middle-aged subjects

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The purpose of Paper III was to investigate the effects of WG rye on cognitive functions and mood, and to further evaluate the metabolic impact of a blend of WG rye flour and rye kernels, supplemented with commercial RS type 2. For this purpose, thirty-eight healthy middle-aged adults (50–70 years old) with normal weight or slightly overweight (BMI 19-28 kg/m^2) participated in a randomized cross-over rye intervention. The rye test product and a WWB reference product were each consumed for 3 consecutive days, separated by a 3-week wash-out period in between. The rye test bread, 'RB+RS', (similar to RFB/RKB+RS in Paper II) consisted of a 1:1 ratio of WG rye flour and rye kernels, and commercial RS2 (Hi-Maize 260) (14% dm). During the first two days of the intervention periods, the daily intake (based on 75 g available carbohydrates) of the test and reference product was ingested freely throughout the day. On day three, i.e. the day prior to experimental day, 50 g of the daily portion was ingested in the evening at 2100 h. The test variables were measured the next morning 11 h after the test or reference evening meal. Cardiometabolic test variables determined at fasting included markers of inflammation, appetite regulation hormones, neuronal integrity (BDNF) and markers of colonic fermentation activity. In addition, markers of glucose regulation and perceived feelings of appetite were measured both at fasting and repeatedly for 3 h in the postprandial period after a standardized breakfast. Mood variables were determined repeatedly during the 3 hour long experimental day. Cognitive performance was evaluated with the use of three working memory tests (WM tests) and two selective attention tests (SA tests).

Results and Discussion

Impact on test variables

In Paper III, it was shown that the RB+RS intervention resulted in significantly increased insulin sensitivity ($ISI_{composite}$) (11%, $P < 0.05$) [90], compared to WWB intervention. Improvements in $ISI_{composite}$ after a 2 h glucose tolerance test have previously been observed after intake of WG rye in individuals with MetS. The study provided a Nordic diet, rich in WG, for 18–24 weeks. Based on a biomarker for relative WG rye intake, a positive correlation between WG rye intake and $ISI_{composite}$ was detected [103]. In Paper III, it was shown that the RB+RS resulted in significantly lower incremental blood glucose response (iAUC) at 0-30 min after the standardized breakfast (-14%, $P < 0.05$), compared to WWB. The incremental insulin response did not differ significantly depending on intervention but the incremental insulin peak value (iPeak) was significantly lower after RB+RS ($P < 0.01$). No differences were observed in blood glucose and insulin fasting value, glucose iPeak values or other postprandial responses.

We observed previously (Paper II) that a RB+RS evening meal decreases postprandial glucose and insulin response (iAUC 0-120 min) in young adults. However, the overnight study design with WG rye products measuring metabolic responses in middle-aged subjects has not previously been applied. In the present study, significant effects were observed on insulin sensitivity but the effects on glucose and insulin response were mainly observed in the early postprandial phase after the standardized breakfast (0-30 min post breakfast or iPeak, respectively), i.e. prior to the start of the cognitive tests. It has previously been shown that peripheral blood glucose concentrations are affected by cognitive processing and, consequently, a period of intensive cognitive load may result in a measurable decrease in glucose concentrations, possibly due to increased neural energy expenditure [104]. In the presently described study it is thus possible that the demanding cognitive tests might have affected the postprandial glucose concentrations. Hence, further studies investigating effects of WG rye products on metabolic responses in middle-aged healthy subjects are needed to confirm this.

The RB+RS intervention resulted in a significant increase in fasting plasma concentrations of GLP-2 (10%, $P < 0.01$) the following morning, compared to WWB intervention. To the best of our knowledge, this study is the first to show such effects of WG rye. GLP-2 has shown to be beneficially involved in gut barrier functions. A well-functioning gut barrier is important as increased gut permeability increases the risk of metabolic endotoxemia and hence also metabolic deteriorations, e.g. increased insulin resistance. It has been observed in mice that the inflammatory tonus was improved after enhancing the production of GLP-2 by consumption of prebiotics (oligofructose) [105]. Thus, inflammatory markers such as lipopolysaccharides (LPS) and cytokines including IL-1 (α and β) and IL-6

were lowered in relation to increased GLP-2 production in prebiotic-fed mice [105].

Recently, it has also been suggested that GLP-2 has an anti-inflammatory effect on the brain, which was observed when mouse microglial cell lines treated with GLP-2 induced lower concentrations of pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) after LPS stimulation compared to no pretreatment with GLP-2 [106]. In the current study it was observed that IL-1β concentrations in plasma were decreased after RB+RS (-11%, $P < 0.05$), compared to WWB. Interestingly, elevated levels of IL-1β have been observed in obese individuals and mice with diet-induced obesity [107]. Furthermore, this pro-inflammatory cytokine was associated with impaired insulin secretion; thus, using IL-1β antibody showed improved glucose control in the obese mice. In the current study no significant differences were observed in concentrations of CRP, IL-6, IL-18 and LBP depending on intervention, and the link between GLP-2, inflammatory tonus and WG rye intake needs to be investigated further.

In comparison to WWB, an increase in the total fasting concentrations of SCFA (i.e. sum of acetate, propionate and butyrate) (32%, $P < 0.001$), and individual SCFA acetate (32%, $P < 0.001$) and butyrate (37%, $P < 0.001$) was observed after RB+RS. However, no significant differences in in propionate concentrations were observed (9%, $P > 0.05$). Together with the increased fasting breath hydrogen excretion (139%, $P < 0.001$), the results show that the DF in the RB+RS product increased the gut fermentation activity. Concomitantly with the increased SCFA after RB+RS, the plasma concentrations of fasting PYY (9%, $P < 0.05$) were significantly increased. The GLP-1 concentrations did not show significant differences depending on intervention (7%, $P > 0.05$). Both PYY and GLP-1 are gut hormones that have been shown to suppress appetite and to possess anti-diabetic potential [108]. However, no significant differences in subjective appetite sensations were observed during the experimental day in the present study.

Effects on cognitive function and mood

The results in the cognitive tests did not show any significant differences depending on intervention. Previously it has been shown that specific changes in diet during a 4-week period have the potential to positively affect cognitive performance in healthy middle-aged subjects. For example, performance in a SA-test was significantly improved after 4 weeks' consumption of a diet composed with the intention of reducing low grade inflammation [109]. Among important food factors in the diet was an increase in WG, such as WG rye and barley. Although the previous study is not directly comparable with the present study due to more changes in the diet, it is possible that a longer duration of intervention than 3 days is needed to observe effects on cognitive performance after intake of WG rye.

Compared to the WWB reference, the RB+RS intervention resulted in significant improvements in the mood parameters, both in valence parameters and activation. Both valence and activation ratings were increased after RB+RS at fasting (9%, $P < 0.05$; 15%, $P < 0.01$, respectively) and during the entire experimental day (0-180 min post breakfast) (10%, $P < 0.001$; 7%, $P < 0.05$, respectively). The mood parameter valence is a mean value based on three rating scales: displeased-pleased, sad-glad and depressed-happy, respectively, indicating higher levels of pleasantness, gladness and happiness after RB+RS compared to WWB. The activation parameter includes means of the three scales: sleepy-awake, passive-active and dull-peppy, indicating higher ratings of feeling awake, active and peppy after the RB+RS intervention. The relation between mood and WG food items has not previously been widely investigated, but intake of a cereal bar at breakfast has shown positive effects on mood parameters directly after intake, determined by increased ratings of happiness and alertness [110]. In addition, previous (unpublished) data has indicated that an evening meal of RKB may induce higher valence in young adults the following morning, compared to consumption of a WWB evening meal. The mechanisms behind the positive effects of rye on mood variables have to be further investigated. However, one possible contributing factor could be the increase seen in PYY concentrations after the RB+RS. Consequently, besides the role of PYY in energy and glucose homeostasis, studies in mice suggest that PYY might have antidepressant effects. Consequently, depression-like behavior was increased in PYY knockout mice [111] PYY has therefore been ascribed a novel role in the so-called gut-mood axis [112]. The results in Paper III showing increased PYY concentrations and a concomitant improvement in mood variables are thus in concordance with previous observations, and suggest possible enhanced mood due to colonic fermentation-induced increase in PYY concentrations.

Correlations

In Paper III, significant relationships were noticed between markers of glucose regulation and cognitive performance. Accordingly, negative correlations were observed between performance in WM tests and insulin iAUC (0-150 min), following both intake of RB+RS (WM:2 & WM:3, $P < 0.05$), and intake of WWB (WM:3, $P < 0.05$ and a trend for the total WM test; WM:1-3, $P = 0.056$). Regarding insulin sensitivity, a significant correlation following RB+RS was seen between $ISI_{composite}$ and the performance in the total WM test (WM:1-3, $r=0.328$, $p=0.044$). In addition, post RB+RS intervention, an inverse correlation appeared between glucose tolerance (iAUC 0-150 min) and the second SA test performed at 170 min after commencing the standardized breakfast ($r= -0.337$, $p = 0.047$). The correlation between WM performance and insulin response observed in the current study is novel; however relationships between performance in cognitive tests and glucose regulation have previously been studied in healthy humans, where subjects

with better glucose regulation performed better in the cognitive tests [31]. The impact of insulin sensitivity on cognitive functions has also been studied in T2D patients with mild Alzheimer's disease, where improving the insulin sensitivity resulted in improved cognitive functions [113].

In addition to the relationships between insulin concentrations and cognitive functions observed in the present paper, relations between insulin concentrations and mood were detected. The most pronounced relationships were seen between insulin concentrations and the mood parameter valence, and among the valence variables it was the subjective happiness rating (depressed-happy) that showed the most distinct relationships to insulin concentrations. Thus, a significant negative correlation was observed between insulin concentrations (iAUC 0-120 min) and the later phase of valence (120-180 min) ($r = -0.339$, $p = 0.04$) post the WWB intervention. In addition, after both interventions (WWB and RB+RS) it was observed that the insulin iAUC 0-120 min response was negatively correlated with happiness (versus depressed) at time point 120 min (WWB: $r = -0.351$, $p = 0.033$; RB+RS: $r = -0.312$, $p = 0.06$). A trend towards an inverse correlation was observed between glucose tolerance (glucose iAUC (0-120 min)) and happiness (depressed-happy) post the WWB intervention ($r = -0.299$, $p = 0.073$).

The mood ratings used in the present study are not used for assessing clinical depression but a more general mood. However, it is of importance to consider mood as an important risk factor for metabolic diseases. For example, studies have shown that there is a relationship between depressed mood, e.g. clinical depression, and increased risk of developing type 2 diabetes, but the underlying mechanisms are still unclear [114].

In conclusion, intake of a WG rye-based evening meal consisting of a blend of WG rye flour and intact kernels, supplemented with commercial RS2 (RB+RS) in the evening, induced higher insulin sensitivity and reduced markers of inflammation (IL-1β) in middle-aged subjects the subsequent morning. In addition, gut hormones involved in appetite and glucose regulation and/or gut barrier functions, PYY and GLP-2, were increased after intake of RB+RS. Upregulation of GLP-2 after rye-based foods in this time perspective is a novel observation and points towards a potential for WG rye to contribute preventive effects with respect to cardiometabolic diseases by lowering of endotoxemia-induced low grade-systemic inflammation. The increase of plasma SCFA concentrations and breath hydrogen excretion after RB+RS suggest involvement of increased gut fermentation activity as a contributing mediator of the beneficial effects observed on the cardiometabolic test variables. Furthermore, intake of RB+RS affected mood variables beneficially the following morning compared to the low-fiber product WWB. Interestingly, measures of cognitive performance and mood were inversely related to insulin concentrations and/or positively related to

insulin sensitivity. Previously, PYY has been proposed to elicit anti-depressive properties, and it can thus be suggested that one contributing underlying factor regarding the beneficial effects of RB+RS on mood seen in the present study may be connected to the concomitant increased concentrations of PYY.

In summary, the presently described study suggests that a WG rye blend (WG rye flour/intact kernels and added RS2) possess anti-diabetic and anti-obesogenic properties in middle-aged subjects, probably mediated through gut fermentation of the DF in the rye product. The relationship between insulin concentrations and/or insulin sensitivity and cognitive functions and mood suggest a preventive potential of WG rye also with respect to cognitive decline due to impaired metabolic control.

Paper IV

Abundance of gut *Prevotella* at baseline and metabolic response to barley prebiotics

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In Paper IV, the intention was to investigate whether the baseline microbiota composition, with focus on the *Prevotella*/*Bacteroides* ratio and abundance of *Prevotella*, could be used to identify and stratify metabolic responders and non-responders to prebiotic mixture present in a barley kernel-based test product. Fecal samples were collected at baseline from a cohort of 99 normal or slightly overweight human donors between 50 and 70 years, with the purpose of characterizing the gut microbiota composition with respect to abundance of *Prevotella* and *Bacteroides*. Thereafter, 33 test subjects with the most pronounced differences in *Prevotella*/*Bacteroides* ratio and abundance of *Prevotella* and/or *Bacteroides* were selected and divided into three groups depending on baseline gut microbiota (‘High *Prevotella*’, HP, n = 12; ’low *Prevotella*’, LP, n = 13; ’high *Prevotella* & high *Bacteroides*’, HPB, n = 8). The subjects included in the different groups then participated in a randomized crossover study consisting of a 3-day intervention with barley kernel bread (BKB) and white wheat bread (WWB, reference), respectively. The daily intake of test and reference product was based on 100 g available carbohydrates. The last portion was ingested in the evening on day 3 and was based on 50 g available starch. The following morning, on day 4, metabolic variables were determined at fasting and during the postprandial period (3 hours) after a standardized breakfast. Test variables in blood included glucose, insulin, gut hormones (GLP-1, PYY, GLP-2), and markers of inflammation (IL-6

and CRP). Measures of insulin sensitivity were also calculated (HOMA, $ISI_{composite}$). Additionally, subjective appetite sensations (hunger, satiety, and desire to eat) and markers of colonic fermentation (breath hydrogen) were determined.

The statistical evaluation of the results investigates the main effects of treatment (BKB and WWB) and baseline gut microbiota, and interactions thereof, using a 2x3 (including all 3 subgroups) or 2x2 (including the 'extreme' groups HP and LP) factorial design. Furthermore, statistical evaluation of metabolic responses to the BKB and WWB, respectively, was performed in each microbiota subgroup separately using one-way ANOVA. See section "Calculations and Statistical Methods" for a more elaborate description of the statistics.

Results and Discussion

In Paper IV, both the incremental postprandial b-glucose responses (iAUC 0-150 min) and the breath H_2 excretion (fasting and mean 0-180 min) at the standardized breakfast showed a main effect of treatment (2x3 factorial design, b-glucose, $P < 0.01$ and H_2, $P < 0.001$). Thus, the results revealed a reduction in b-glucose response and an increase in breath H_2 concentration post BKB intervention, compared to WWB intervention, i.e. independently of gut microbiota composition. No other main effects of treatment were detected regarding other test variable responses (2x3 factorial design, $P > 0.05$).

The statistical evaluation of treatment effects on test variables in each subgroup separately (using one-way ANOVA) displayed that the fasting b-glucose concentration was significantly increased by 6% in the LP group the following morning after 3 days of BKB intervention ($P < 0.05$), compared to WWB intervention. No such observations were detected in the subgroups HP and HPB ($P > 0.05$). Furthermore, the postprandial s-insulin responses (0-150 min, AUC and iAUC) in groups HP and LP were significantly decreased after BKB intervention (one-way ANOVA, $P < 0.05$), compared with WWB intervention. The gut hormone concentrations of GLP-1 and GLP-2 were increased in the HPB group after BKB (one-way ANOVA, $P < 0.05$), whereas there was a strong trend towards increased PYY concentrations in the HP group post BKB intervention (one-way ANOVA, fasting, $P = 0.050$ and mean 0-180 min, $P = 0.060$). Measures to increase gut hormones is probably important with respect to cardiometabolic health, thus, in addition to the satiety-inducing properties of PYY and GLP-1, these gut hormones have been ascribed anti-diabetic potential [108]. and GLP-2 is related to enhanced gut barrier functions, and has been assigned with anti-inflammatory properties [115]. However, concentrations of p-IL-6 and p-CRP showed no significant differences due to treatment in any of the separate microbiota subgroups ($P > 0.05$). The appetite sensations were significantly

improved in the LP group after the BKB with respect to decreased sensation in hunger and increased satiety at fasting (one-way ANOVA, $P < 0.05$ and $P < 0.01$, respectively) and a trend towards reduced postprandial desire to eat (one-way ANOVA, AUC 0-180 min, $P = 0.082$), compared to the WWB intervention.

No treatment effects on other test variables ($ISI_{composite}$, HOMA-IR or FFA) were displayed when analyzing the subgroups separately (one-way ANOVA, $P > 0.05$).

The results in Paper IV, with respect to metabolic influences of barley compared to a reference WWB, did not show any major differences between the microbiota subgroups, and all subgroups responded to BKB with improved glucose tolerance 11 h after intake, compared to WWB. Thus, the hypothesis that the ratio *Prevotella*/*Bacteroides* at baseline can be used to identify and stratify individuals that are responders to barley from non-responders in a population could not be confirmed [13]. The metabolic effects of barley kernel-based products were in accordance with previous results showing beneficial effects on glucose and insulin response, gut hormones (GLP-1, GLP-2 and PYY), subjective appetite variables, and increased breath H_2 concentrations [11, 34, 44, 116]. However, previous observations regarding reduced IL-6 and FFA concentrations after BKB intake were not confirmed [11, 34, 44]. In addition, increased fasting concentrations of b-glucose, as observed in the LP group, has not previously been observed in cohort that were randomly selected, i.e. not on the basis of baseline microbiota composition.

Interestingly, the metabolic responses in Paper IV did display some main effects of baseline gut microbiota composition independently of treatment. Firstly, when including all three subgroups (HP, LP and HPB), i.e. the 2x3 factorial design, a main effect of baseline gut microbiota composition was detected on appetite variables (AUC 0-180 min, hunger, $P < 0.05$ and desire to eat, $P < 0.01$) and breath H_2 levels (fasting and mean 0-180 min, $P < 0.05$), and a trend towards a main effect of microbiota composition was observed on the s-insulin responses (iAUC 0-150 min, $P = 0.070$). The post hoc analysis (Tukey method) showed that the LP group had significantly higher sensations of hunger and desire to eat (AUCs 0-180 min) compared to the HP group (hunger, $P < 0.05$; desire to eat $P < 0.01$) and the HPB group (hunger and desire to eat, $P<0.05$). Moreover, results from the Tukey method displayed that the fasting H_2 concentrations were higher in the LP group compared with the HP group ($P < 0.05$), indicating differences between the two groups with respect to gut microbiota activities. The post hoc analysis did not show any significant differences in the insulin responses between microbiota subgroups (Tukey method, $P > 0.05$)

Statistical evaluation was also performed without the HPB group to facilitate the elucidation of differences between the "extremes" with respect to the abundance of *Prevotella* (HP versus LP). Thus, by limiting the subgroups to HP and LP

subgroups (2x2 factorial design), a main effect of baseline gut microbiota was shown on s-insulin response (iAUC 0-150 min, $P < 0.05$) and mean concentrations of p-IL-6 (mean 0-180 min, $P < 0.05$) following the standardized breakfast. Furthermore, a trend towards a main effect of baseline microbiota composition was noted at fasting CRP ($P = 0.084$). Thus, the results revealed that the HP group showed an overall reduced insulin response, IL-6 concentrations and trends towards lower CRP concentrations compared to the LP group. Consequently, it is suggested that a higher abundance of *Prevotella* and higher *Prevotella*/*Bacteroides* ratio at baseline is advantage with respect to insulin economy and systemic inflammation tone.

No significant main effects of gut microbiota composition at baseline were observed (2x3 and 2x2 factorial design) with respect to $ISI_{composite}$, HOMA-IR, FFA or the gut hormones (PYY, GLP-1 or GLP-2). However, it is noteworthy that after the WWB intervention, i.e. without intake of barley prebiotics, the GLP-1 concentrations in the HP group were 26% and 33% higher compared to the GLP-1 concentrations in the LP and HPB subgroups ($P > 0.05$), respectively. Furthermore, after the BKB intervention the HP group had 18 % higher (not significant) GLP-1 concentrations compared to the other two subgroups ($P > 0.05$). Thus, the results in Paper IV indicate significant differences in cardiometabolic test markers between the microbiota subgroups independently of intervention. However, the results in this study, focuses on the impact of gut microbiota at baseline and do not exclude that subjects that respond beneficially to BKB have an expansion of *Prevotella* after the BKB intervention, regardless of baseline microbiota composition, and hence, that the increase in *Prevotella* after BKB mediates the improved glucose metabolism, similar to what was found in the previous report [13].

The systemic effects of gut *Prevotella* have not been investigated extensively in intervention studies, and observational studies reporting relationships between gut abundance of *Prevotella* and systemic health are scarce. However, there are reports showing inverse relationships between abundance of gut *Prevotella* and prevalence of diabetes (type 1 [117, 118] and type 2 [119], respectively), Parkinson's disease [120], multiple sclerosis [121], and autism associated gastrointestinal problems [122]. In addition, *Prevotella* is known to produce succinate and it has recently been suggested that succinate, as produced from *Prevotella*, may be used as a substrate in intestinal gluconeogenesis, resulting in improved glucose homeostasis [61]. In contrast, it has been discussed by others that succinate is involved in the signaling of inflammation [123]. Some other studies (observational) have observed correlations between a higher abundance of *Prevotella* (*P. copri*) in patients with early onset of rheumatoid arthritis (RA) compared to chronic RA patients and/or healthy controls, indicating involvement of *Prevotella* in certain inflammatory conditions [124, 125]. However, the results

in Paper IV do not indicate a relationship between inflammatory markers and abundance of *Prevotella*. On the contrary, higher abundance of *Prevotella* at baseline tended to predict an overall lower inflammatory tonus, indicated by the significantly lower concentrations of IL-6 and trends towards lower CRP levels in the HP group.

In Paper IV, although 33 subjects were included, the number of subjects in each arm was low, which is a limitation of the study. The purpose was to select two groups (HP and LP) with 20 subjects in each group from the total of 99 subjects, however, it was not possible because a clear cut-off could not be distinguished between 20 subjects with a high abundance of *Prevotella* and low abundance of *Bacteroides*, and vice versa. However, a third group subjects with relatively high levels of both *Prevotella* and *Bacteroides* (the HPB group) were identified and these subject were included as a potentially interesting additional group to study as taxa belonging to these genera usually are believed to compete for the same niche [126].

In conclusion, the results suggest an overall anti-diabetic potential of BKB independently of *Prevotella*/*Bacteroides* ratio at baseline. Consequently, the results did not support the hypothesis regarding using baseline *Prevotella*/*Bacteroides* ratio as a strategy to identify the individuals who would acquire metabolic benefits from the prebiotic mixture present in barley kernel products. Instead, in Paper IV, reduced insulin responses and inflammatory markers (IL-6 and CRP) and improved appetite ratings in the HP group, suggest that higher abundance of gut *Prevotella* and *Prevotella*/*Bacteroides* ratio at baseline may be beneficial with regards to cardiometabolic regulation. Thus, the results in Paper IV support previous observations of the anti-diabetic potential of barley prebiotics but in addition it is indicated that higher *Prevotella*/*Bacteroides* may play a preventive role in development of obesity, T2D and CVD.

General Discussion

A diet rich in WG and DF has been ascribed to have preventive potential against obesity, T2D and CVD. These metabolic disorders are further associated with an increased risk of cognitive decline and depression. The role of gut microbiota in health and disease, and the impact of diet have been increasingly highlighted throughout the years. In this context, the work included in this thesis provides novel insight into WG rye-based products rich in fermentable DF (especially rye kernels or a mixture of kernels, flour and RS), and baseline gut microbiota composition in relation to glucose and appetite regulation in healthy subjects. The results indicate that intake of WG rye kernels solely or combined with WG rye flour and commercially available RS2 can be useful in the prevention of lifestyle-related metabolic disorders, i.e. obesity, T2D and CVD.

The studies included in this thesis have involved interventions with food products differing in botanical structures, ranging from mainly kernel-based products in the case of rye and barley, to mixtures of kernels and flour, and flour-based products in the case of rye. In Paper I, the choice to include rye kernel-based products in the overnight study design was based on the fact that the prebiotic potential in rye has not been studied before and on reports from previous studies with similar study design showing that the structure of WG foods may impact the glucose tolerance. Thus, inclusion of products based on intact kernels of barley as an evening meal importantly enhanced glucose tolerance the following morning. In contrast, an evening meal consisting of WG barley flour-based porridge did not [33]. However, as the choice of kernel ingredient limits the range of product applications, the present thesis set out to investigate whether it is possible to design test products with lower concentrations of kernels and higher levels of WG rye flour still maintaining anti-diabetic potential. Therefore, in Paper II a product with a 1:1 mixture (dm) of WG rye flour and rye kernels (RFB/RKB), and a product solely based on WG rye flour (RFB) were included. However, as the milling process of kernels to flour leads to loss of RS, mainly in the form of RS1, RS type 2 was included to compensate for the lost RS. Results in Paper II showed that supplementation of RFB (RFB+RS) with 25% RS2 did not affect glucose and insulin response in comparison to a WWB reference product, and it is suggested that high concentrations of commercial RS2 might dilute the endogenous DF in rye due to the relatively high (40%) amounts of available starch. Instead, 14%

supplementation of the RFB/RKB product with RS2 (RFB/RKB+RS) resulted in a similar (or even slightly enhanced) reduction in glucose and insulin responses in healthy young adults compared with the responses after RKB in Paper I. This indicates the potential of using moderate amounts of RS2 to enhance the metabolic benefits of a rye-based product with a higher ratio of WG rye flour. Thus, in future studies it would be interesting to investigate whether similar improvements in metabolic responses can be mimicked following supplementation of RFB with a moderate amount of RS2. The results in Papers I and II demonstrate that several different structures of rye (e.g. both intact kernels and rye flour) may provide beneficial effects in healthy individuals. This result is important in that it increase possibilities for development of a high variation of food products with anti-diabetic potential to provide alternatives for consumers to facilitate increased WG intake.

Intake of RKB (Paper I) and RFB/RKB+RS (Paper II) decreased the glucose response by 23% and 27%, respectively, and the insulin response by 13% and 21%, respectively (iAUC 0-120 min) in young healthy adults, compared to WWB. Barley kernels have also displayed anti-diabetic effects in healthy young adults in several previous intervention studies using similar study design. Consequently, based on four studies including young adults and standardized breakfast, the average decrease in postprandial glucose response (iAUC 0-120 min) post the barley kernel evening meal was 34% (ranging from 28% to 46%) [33, 34, 44, 127], whereas the postprandial insulin response after barley kernel intake varied from 19% to 33% [34, 44], compared to WWB or glucose drink. This indicates that barley kernels had somewhat more advantageous properties on glucose tolerance compared with the rye kernel-based products in Paper I and Paper II. In addition to improved glucose tolerance, barley kernels, compared with the rye products in the present thesis, displayed a higher percentage in enhancement of GLP-1 in healthy young adults [11, 34]. Instead, rye kernel-based products in this thesis induced higher PYY concentrations (Papers I and II) and more improved appetite variables (Paper I), compared to barley kernel-based products [11, 128]. Thus, since both barley and rye kernel-based products affect glucose tolerance and appetite regulation beneficially but slightly differently, it would be interesting to observe wheter combining these different cereals, rich in different DF types such as β-glucans (barley) and arabinoxylans (rye), would enhance the existing beneficial cardiometabolic effects, possibly in a synergistic manner.

This thesis revealed that WG rye kernels solely or combined with WG rye flour and RS as evening meals have anti-diabetic potential, i.e. by decreasing glucose and insulin responses (Papers I, and II) or increasing insulin sensitivity (Paper III) at a standardized breakfast the following day. Furthermore, evening meals with rye-based products have shown simultaneous enhancement of perceived appetite sensations (Papers I and II) and increased gut hormones (GLP-1, PYY and GLP-2)

involved in appetite- and glucose regulation and/or gut barrier functions (Papers I-III), the following morning. Furthermore, it was found that a mixture of WG rye kernels, flour and RS can improve the mood, e.g. happiness and wakefulness, in healthy volunteers. This is of importance as declines in mood such as depression are linked to an increased risk of, for example, T2D [129]. Additionally, it was observed that fasting SCFA was increased 11 h post the evening meals with RKB and RB+RS (Papers I and III), and that all WG rye products (kernel and/or flour-based) increased postprandial H_2 excretion 11–14 h after intake (Papers I-III), in comparison to WWB. These results indicate increased gut fermentation due to the DF in rye products. Based on the results in this thesis it is suggested that the improved metabolic effects in the overnight perspective investigated are due to gut fermentation of the DF in the rye products (endogenous DF and supplemented RS2). Thus, the anti-diabetic and anti-obesogenic potential observed after intake of rye, based on the beneficial effects on glucose tolerance and subjective appetite ratings, are suggested to involve the increased release of the gut hormones GLP-1 and PYY, which are known to play an important role in glucose and appetite regulation. Gut fermentation of the DF in the rye products increased formation of the fermentation metabolite SCFA. It has been shown in *in-vitro* studies that the SCFA stimulate secretion of GLP-1 and PYY [130]. Consequently, positive correlations were observed between fasting levels of SCFA (total, acetate and propionate) and the postprandial concentrations of GLP-1 following the standardized breakfast after RKB (Paper I).

Correlations between gut fermentation markers and improved glucose regulation have also previously been observed. For example, in a day-long study when test products were consumed at breakfast, a negative correlation was observed between the H_2 excretion and glucose response (iAUC) at the standardized dinner. Consequently, the reduced glucose response observed at a standardized dinner was suggested to be mediated via mechanisms derived from gut fermentation of the DF in rye and barley, respectively, consumed at breakfast [33]. In addition, plasma levels of acetate, propionate and butyrate correlated inversely with blood glucose regulation at breakfast in overnight studies providing barley kernel products as evening meals [127, 131]. In addition to DF, it should be noted that the WG rye also contains several other potentially bioactive components, e.g. alkylresorcinol, phenolic acid and lignans, which may protect against cardiometabolic disease and contribute to the beneficial effects of fiber-rich foods [132]. However, based on the results in the present work and previous studies including similar study design, it is suggested that gut fermentation of DF in rye constitutes an important mechanism.

In addition to the PYY and GLP-1, the concentrations of gut hormone GLP-2 were measured after intake of rye-based test products in Paper I and Paper III, and after intake of barley kernel-based products in Paper IV. A significant increase in GLP-

2 concentrations occurred in middle-aged subjects (Papers III and IV) after RB+RS and BKB, respectively, but no differences were observed after RKB versus WWB in Paper I. Information regarding the impact of food on GLP-2 in humans is scarce, thus the increase in GLP-2 concentrations after WG rye intake (RB+RS) in this time perspective is novel (Paper III), and the results add important new information. It has been shown that GLP-2 has a positive impact on intestinal epithelial barrier. Gut permeability increases with age [133], and an increase in GLP-2 concentrations (as seen after intake of RB+RS in Paper III) could thus be of major importance in middle-aged subjects. GLP-2 is also regarded as anti-inflammatory, and in accordance with increased plasma GLP-2 levels in Paper III, there was a concomitant decrease in the inflammatory marker IL-1β. No significant effects were observed on other markers of systemic inflammation.

This thesis has also included different aspects of the gut microbiota composition. In Paper I the focus was on the alterations in microbiota composition depending on treatment (rye kernels or WWB), and in Paper IV the focus was to investigate whether the metabolic response to barley could be predicted depending on baseline gut microbiota composition with respect to abundance of *Prevotella* and *Bacteroides*. Interestingly, it was observed that significant alterations in gut microbiota composition on family and genus level occurred quickly (Paper I). The results indicate that the gut microbiota composition changes dynamically with diet. In contrast, the *basal* gut microbiota composition, i.e. without interference with specific diet intervention, has been shown, by others, to remain relatively stable over time. This was reported after observations revealing that the microbiota composition was stable in subjects in a one-year perspective, accompanied with stable metabolic responses, e.g. glucose tolerance, in the same time perspective [13]. In addition, it was noticed that subjects with a higher ratio of *Prevotella* to *Bacteroides* acquired increased benefits on glucose tolerance in comparison with subjects with a low ratio of *Prevotella*/*Bacteroides* [13]. Therefore, in this thesis it was relevant to examine whether differences in metabolic responses to barley could be predicted from baseline gut microbiota composition with respect to *Prevotella*/*Bacteroides* ratio. The results showed that, independently of the baseline microbiota composition (high versus low ratio of *Prevotella*/*Bacteroides*), beneficial effects on glucose regulation were observed after barley kernel products. The results thus indicate that barley prebiotics may affect certain metabolic responses independently of microbiota composition in healthy subjects. However, a higher abundance of baseline *Prevotella* resulted in overall lower insulin concentrations, lower IL-6 levels and improved subjective appetite sensations (hunger and desire to eat). However, this study was performed in a small study population and, accordingly, further studies are needed to confirm these results.

Taken together, the thesis provides novel insights regarding the anti-diabetic and anti-obesogenic potential of WG rye-based products (especially rye kernel-based or WG rye kernel and flour-based with RS2). This was indicated by improved glucose tolerance, enhanced concentrations of gut hormones involved in glucose and appetite regulation and gut barrier functions, and improved subjective appetite ratings post intake of WG rye-based products. Based on the increase in SCFA and breath H_2 concentrations after WG rye, the effects are suggested to be mediated via gut fermentation of DF in rye, and possibly by a rapid alteration of the gut microbiota composition towards a more beneficial composition with respect to metabolic health post intake of rye kernels. In addition, the results regarding beneficial impact on mood by the product including a blend of WG rye flour, rye kernels and RS, further emphasizes the interplay between the gut-brain axis and diet, see illustration of suggested underlying mechanisms **Figure 5**. Lastly, the thesis also shows potential differences in metabolic outcomes depending on baseline gut microbiota composition, adding to the discussion regarding future possibilities for personalized nutrition. These observations provide new knowledge that can be used to prevent metabolic disorders such as obesity, type 2 diabetes and cardiovascular disease.

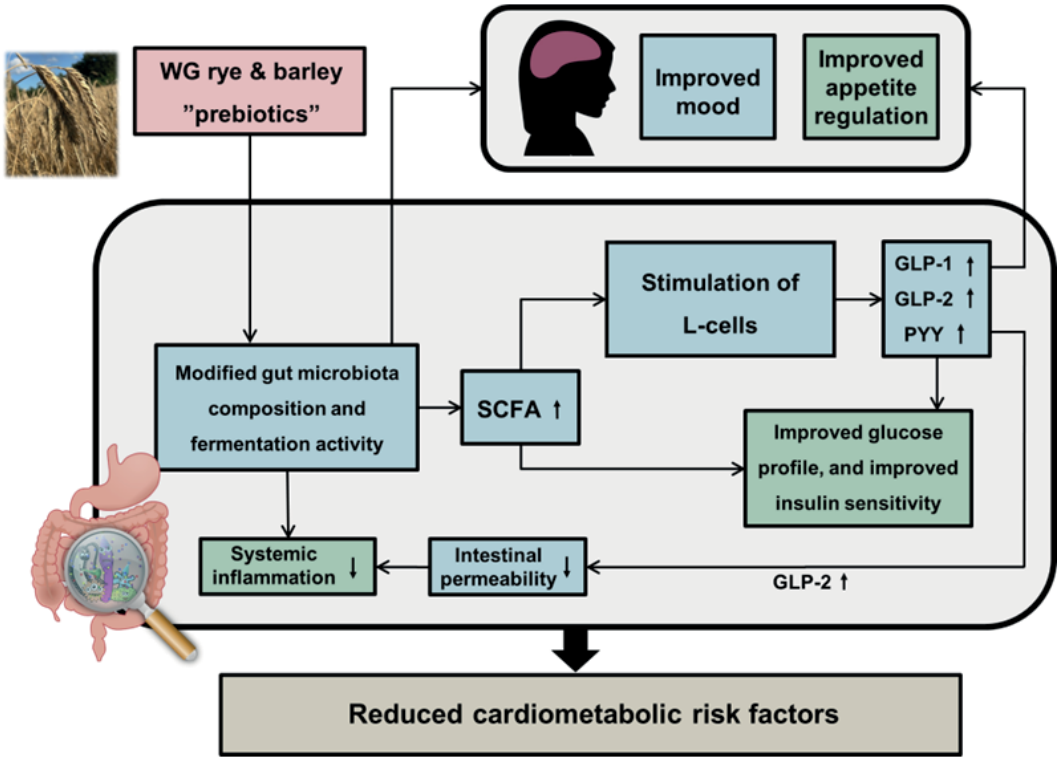

Figure 5. Suggested underlying mechanism based on main findings in the present thesis work.
Suggestion of mechanistic pathways for prebiotic effects of rye and barley-based products on metabolic and appetite regulation, and regulation of mood in healthy humans. Higher ratio of *Prevotella*/*Bacteroides* at baseline is suggested to beneficially impact on parameters in the green boxes.

Conclusions

The main conclusions from the present thesis are;

- Evening meals with kernel-based rye breads, i.e. RKB and RFB/RKB+RS, improved glucose tolerance and insulin economy at a standardized breakfast the next morning in young adults, and improved insulin sensitivity in middle-aged individuals, indicative of anti-diabetic properties of such products.
- No significant effects were detected on glucose regulation with WG rye flour-based breads, with or without RS supplementation. The results indicate the importance of maintaining a certain degree of botanical structure, hence contributing with RS in the form of botanically encapsulated starch.
- The breads RKB, RFB/RKB and also the RFB improved subjective appetite sensations in a time perspective of approx. 11–14 h post intake. Thus, in addition to anti-diabetic properties, RKB, but also the RFB/RKB and RFB possess anti-obesogenic potential.
- Breads including intact kernels, i.e. RKB, RFB/RKB+RS, increased GLP-1 and/or PYY in plasma. These gut hormones are important in glucose- and appetite regulation. No increase was observed after 100% WG flour bread, with or without RS supplementation, i.e. RFB and RFB+RS, despite improvements on appetite variables in the case of RFB.
- Increased plasma levels of GLP-2 and reduced pro-inflammatory cytokine IL-1β concentrations were observed in middle-aged subjects after bread made from a combination of WG rye flour, rye kernels, and with RS2 supplementation (RB+RS), indicating an anti-inflammatory potential.
- As indicated by increased abundance of *Prevotella* and *Fecalibacterium*, and decreased abundance of *Bacteroides*, rye kernel-based products induced rapid alterations in gut microbiota composition. No significant differences in gut microbiota composition were observed depending on length of priming (1 day or 3 days).
- An evening meal with RB+RS affected mood parameters positively, as judged from improved valence and activation variables. The results thus

indicate that also mood parameters may be improved through a prebiotic mechanism.

- Mood and measures of cognitive performance were positive associated with glucose regulation and/or insulin sensitivity. The results suggest a direct relationship between metabolic and cognitive health in healthy subjects. The results propose that cognitive impairments due to metabolic disorders can be prevented by a diet displaying anti-diabetic properties. WG rye may be potential in this respect.
- Independently of baseline abundance of *Prevotella* and *Bacteroides*, individuals in the study cohort benefited from intake of barley kernel-based bread in that they improved glucose regulation at a subsequent standardized breakfast.
- A high *Prevotella*/*Bacteroides* ratio at baseline, i.e. prior to any diet intervention, was accompanied by lower postprandial insulin response, lower inflammatory tone (IL-6 and CRP) and improved subjective appetite variables, compared to a low baseline *Prevotella*/*Bacteroides* ratio. The results indicate involvement of gut microbiota composition in metabolic regulations in favor of a high *Prevotella*/*Bacteroides* ratio.
- Intake of RFB, RKB, RFB/RKB, RFB+RS, RFB/RKB+RS, RB+RS and BKB bread increased markers of colonic fermentation, i.e. breath H_2 and/or plasma SCFA concentrations in young as well as in middle-aged subjects, respectively.
- SCFA concentrations were positively correlated with concentrations of gut hormone GLP-1.

In conclusion, the results in this thesis provide novel knowledge regarding the prebiotic beneficial potential of WG rye products (especially rye kernel-based or WG rye kernel and flour-based with RS2) on systemic effects on cardiometabolic risk markers and appetite regulation, and also on mood parameters. Finally, the thesis suggest that baseline abundance of *Prevotella* and *Prevotella*/*Bacteroides* ratio affect systemic metabolism, and measures to increase gut *Prevotella* may be advantageous with respect to cardiometabolic health.

Future Perspectives

In future studies, it would be interesting to investigate the metabolic effects of WG rye in individuals with metabolic disorders such as obesity, MetS, T2D and CVD, to observe whether these individuals are also beneficially affected by the rye-based products included in this thesis.

Moreover, studies including longer intervention periods (weeks or months) of rye-based products are of importance to further investigate the relationship between metabolic responses, mood and WG rye in healthy subjects and subjects at risk or with metabolic disorders. In addition, future perspectives could include observing whether the positive impact of mood after intake of WG rye can be obtained in other cohorts comprising other ages than middle-aged individuals, as declined mood is an issue for all ages. It would be of interest to observe whether WG rye may have a positive impact on cognition in longer term interventions. Studies including a more realistic breakfast composition would be useful to investigate the effect of WG rye products on energy intake, as WG rye products showed anti-obesogenic potential, as indicated by increased satiety-inducing hormones and improved subjective appetite ratings.

Based on the suggestion that higher concentrations of commercial RS will dilute the endogenous DF in rye due to high levels of available starch (40%), increased knowledge regarding how to manufacture resistant starch with a low content of available starch would be of importance.

It would also be important to further investigate the impact of baseline gut microbiota composition, e.g. by including more subjects, and/or longer-term interventions, to confirm the suggested advantage of high gut *Prevotella*/*Bacteroides* ratio with respect to metabolic responses such as insulin economy, inflammation tone and appetite regulation. Furthermore, future studies should also aim to increase the knowledge regarding optimization of prebiotic substrates for optimal metabolic effects on metabolism. Thus, by identifying which bacterial species are amplified and by identifying causal relationships between microbiota metabolism and the metabolic responses in humans it may be possible to establish and identify probiotics and to tailor prebiotic, probiotic and synbiotic food products with anti-diabetic and anti-obesogenic properties.

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