Sveriges lantbruksuniversitet
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Faculty of Natural Resources and Agricultural Sciences
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Magnus Lindelöf

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Magnus Lindelöf

Supervisor: Dr Carl Brunius, Swedish University of Agricultural Sciences, Department of Food Science

Assistant Supervisor: MSc, PhD Candidate, Lin Shi, Swedish University of Agricultural Sciences, Department of Food Science

Examiner: Dr Galia Zamaratskaia, Swedish University of Agricultural Sciences, Department of Food Science

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Abstract

Rye products are known to more effective decrease different aspects of subjective appetite compared to low fiber wheat bread. Nutritional composition and structural properties, such as dietary fiber components and grain integrity are thought to play roles in the satiating mechanism behind this appetite reduction. The aim of this master project was to investigate how a whole grain rye porridge breakfast with addition of plant protein and easily fermented fiber is reflected in the short term plasma metabolome and if there are differences in the metabolome that may explain differences in perceived appetite in healthy people.

The study design was a single blinded randomized cross-over with six treatments tested on healthy men and women (n = 21). The tested meals were iso-caloric servings of plain rye porridge made of 40 g rye (R), 55 g rye (R55), or R with three levels of easily fermented dietary fiber and plant protein; 9 g fiber/ 3 g protein (RHF), 6 g fiber/6 g protein (RPF), and 3 g fiber/9 g protein (RHP). White wheat bread (WWB) served as control. Plasma metabolome was studied at 8 time points over an 8 hour period containing a standardized lunch and a dinner with unlimited supply. Perceived appetite was assessed with visual analogue (VAS) scale every 30 minutes.

Untargeted NMR-based metabolomics was applied on ultra-filtrated, protein free, plasma samples. To facilitate the primary aim the automated NMR peak picking and alignment treatment methods of “speaq” (Spectral Alignment and Quantification) package in the software programming language R were developed and evaluated. Prior to “speaq” processing NMR data were subjected to traditional manual phase and baseline correction in TopSpin™ 2.0 (Bruker Corporation, USA). The processed NMR dataset was analyzed with principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) to seek correlations between the metabolome and the rye breakfasts.

RHF, RPF, RHP and R55 were found to reduce the feeling of hunger over 8 hours (Least Square Means) compared to WWB. The final data treatment workflow resulted in > 1200 detected features. Of those, a vast quantity was noise and many peaks were missing, but still a lot of useful information was generated in a fraction of the time required for current manual peak picking methods. PCA and PLS-DA models on entire dataset or subsets were distinguished by time points, individuals, or baseline differences between occasions, rather than by treatment. The current methods could not distinguish between metabolomes of any of the six treatments.

In this project several important steps towards an automated processing of NMR data were made. Further research on improvement of the “speaq” workflow in general and peak picking in particular are suggested, as well as accurate assessment in the statistical models of the large variation not related to the diet intervention.

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Abbreviations

BMI	Body Mass Index
E %	Energy Percentage
FOS	Fructooligosaccharides
HMDB	Human Metabolome Database
NMR	Nuclear Magnetic Resonance
PC	Principal Component
PLS-DA	Partial Least Squares Discrimination Analysis
R	Rye 40 g
R55	Rye 55 g
RHF	Rye High Fiber
RPF	Rye Protein Fiber
PCA	Principal Component Analysis
RHP	Rye High Protein
SCFA	Short Chain Fatty Acid
SPEAQ	Spectral Alignment and Quantification
T2D	Type II Diabetes
TSP	Trimethylsilyl Propionic Acid
VAS	Visual Analogue Scale
WWB	Refined Wheat Bread

Background

Rye products is more effective than wheat products in ability to increase satiety, reduce hunger, desire to eat and postprandial insulin response (Leinonen *et al.*, 1999; Juntunen *et al.*, 2003; Isaksson *et al.*, 2009; Rosen *et al.*, 2009). The underlying reasons and mechanisms behind these effects remain to be fully elucidated. Rye diets are often characterized by high dietary fiber (DF) content. Dietary fiber plays a role in satiety through different mechanisms. One mechanism is intestinal fermentation which has been suggested to affect appetite (Nilsson *et al.*, 2008a; Cani *et al.*, 2009). The protein composition is also of interest in the effect on satiety since proteins are known to be more satiating than equal amounts of carbohydrates or fat. To investigate the current standing on appetite effects of rye products, a database literature search limited to human studies was performed using PubMed to cover and select relevant publications. In total, 224 original research articles corresponded to the complete search question; (satiety) AND (rye OR (fiber OR fibre) OR (ferment*) OR "dietary protein"). Articles regarded as relevant for the research questions in this thesis were used to describe the background.

Appetite

Satiety and appetite can be described as the variation in psychological and physiological response to food intake or periods of starvation (Blundell *et al.*, 2010). According to Graaf et al., (2004) this phenomenon can be further divided into satiation, i.e. meal terminating responses and satiety which refers to effects on meal initiation in the postprandial and starvation periods. The broader term appetite is used when discussing subjective responses to food intake or starvation in more general terms and may refer to effects on satiety, hunger and desire to eat.

Appetite clearly influences our eating patterns and may partly explain between-individuals sometimes eat more than their physical requirements and become overweight. Appetite and food intake are linked to physiological responses such as gut hormones and peptides (e.g. ghrelin, glucagon like peptide 1 (GLP-1), chole-

cystokinin (CCK), peptide YY) (Moran, 2009). The food metabolism is moreover regulating secretion of insulin in response to carbohydrates and leptin to starvation. All these substances are known to affect appetite, but the whole complex of triggers and responses is not well understood. Furthermore, if psychological factors are taken into account, appetite prediction or assessment becomes a multidisciplinary issue with many possible biasing factors. The physiological and psychological factors probably interact for the effects on appetite. One way to dissect the physiological meal related effects of appetite could be to use untargeted studies of events in the blood in order to link differences in subjective appetite to changes in the composition of the blood.

Rye foods

Rye is a cereal of importance in the diet in Northern Europe and national supply of rye is highest in the countries in the Baltic region with an extension to Belarus, Ukraine and the Czech (FAOSTAT, 2009). The traditional use of rye in this region has made it an interesting marker and distinguisher of a typical "Nordic diet" (Bere & Brug, 2009). One feature that makes the Nordic diet and rye interesting, and possibly beneficial for health, is that rye products, compared to low fiber wheat products reduce appetite, as shown in several short term intervention studies (Leinonen *et al.*, 1999; Juntunen *et al.*, 2003; Isaksson *et al.*, 2009; Rosen *et al.*, 2009). Rye does not only exert its satiating effect in the immediate postprandial phase, but can affect satiation in later meals. A single breakfast with either wholegrain rye porridge (Isaksson *et al.*, 2008) or rye bread (Isaksson *et al.*, 2009) significantly reduced appetite compared to a white wheat bread (WWB) control serving both in the period from breakfast until lunch and in the afternoon after a standardized lunch meal without rye. In the study with rye porridge, the reduced appetite persisted until 8 hours after the breakfast, but did not decrease the energy intake at the *ad libitum* dinner (unlimited supply of food to be eaten until feeling comfortably full) served at that time point (Isaksson *et al.*, 2008). The sustained reduction in appetite after lunch is known as "second meal" effect.

Both genetic variation and environmental factors of rye, both with potential to alter the chemical composition of the grain, may play a role for the effect on appetite as suggested by recent interventions (Rosen *et al.*, 2011a). Two of the tested rye varieties were found to be correlated with both lower insulin and glucose level in early postprandial period (<60 min) and were linked to reduced appetite compared to WWB. Differences in fiber and phenolic compounds may explain the differences to some extent. Assessment of differences caused by cultivar on appetite is necessary if health claims are to be coupled to rye or rye products in commercialization and also to aid in improvement of public health advices.

Dietary fibre, colonic fermentation and satiety

Rye is generally consumed as whole grain and is high in DF. High DF foods has been associated with beneficial effects on health (Liu, 2007). According to American Association of Cereal Chemists International (AACCI) DF is defined as the indigestible and fermentable carbohydrates, lignins, and associated substances of the edible parts of a plant. In rye the DF is characterized by the high content of fructans and soluble arabinoxylans, indigestible, but readily fermented by colonic microflora (Broekaert *et al.*, 2011). The DF that escapes small intestine is metabolized by colonic microflora to short chain fatty acids (SCFA) that are absorbed by the intestine. SCFA contribute with energy to the colonocytes, but also reach the systemic circulation to some degree and is therefore part of the plasma metabolome (Wong *et al.*, 2006). Soluble fibers absorbs water that contribute to an increased bulking effect that can reduce appetite (Rolls *et al.*, 1998). The lower energy density of foods rich in DF will not reduce appetite, but reduce the ingested amount of energy. A slower gastric emptying rate of DF are also associated with reduced appetite (Benini *et al.*, 1995).

Food structure may also affect appetite, e.g. coarseness of the flour used. The intact structure of a rye porridge breakfast with kernels may contribute to a second meal effect and reduce post-lunch appetite compared to plain rye porridge (Isaksson *et al.*, 2011). However, this difference in appetite between kernels and wholegrain flour was not observed in a bread matrix in the same study. The structural component is also addressed by Rosén et al., (2011b) in breakfasts with boiled rye kernels or wholegrain rye bread, made from flour. They found decreased appetite ratings and reduced energy intake at a subsequent lunch for both the kernels and the wholegrain rye compared to control white bread and the appetite reducing effect was especially good with the boiled kernels. They suggested the satiation and early satiety effects to come from bulking while the later satiety was caused by colonic fermentation. This was stated despite that colonic fermentation was negatively correlated to energy intake and not correlated to decreased desire to eat in the late postprandial period. After a whole grain rye porridge breakfast, fermentation starts within 240 minutes with a high fermentation peak at 300 minutes in persons subjected to a rye diet for one week (Isaksson *et al.*, 2012). This fermentation pattern was not followed by decreased satiety ratings and colonic fermentation is suggested to be of less importance in the short term satiating mechanism of rye. However, the relation between colonic fermentation and appetite in cereal diets may not be completely obsolete. Bread with barley kernels, rich in fermentable β-glucans, served as “preload” (a serving prior to a standardized main meal) 10 hours in advance of a breakfast increased fermentation during a 180 minutes postprandial period compared to WWB (Nilsson *et al.*, 2008b). The increased rate of colonic fermentation was in this case positively correlated to de-

creased appetite ratings. If less energy is absorbed from ingested foods, maintenance of a sound energy balance is easier to achieve. Thus, reduction of the amount of energy that actually is absorbed in the small intestine is of interest. After three days of high fiber rye diet, compared to low fiber wheat diet, in persons with illelal stomy, more undigested nutrients were excreted from ileum (Isaksson *et al.*, 2013).These nutrients may contribute to colonic fermentation and was estimated to lower energy uptake in ileum with 0.2 MJ per day. The authors relate this lower energy utilization to arabinoxylans and their observed effect on energy uptake in previous animal studies (Pettersson & Åman, 1988; Andersson *et al.*, 2010).

Easily fermented fibers, such as inulin and fructooligosaccharides (FOS), may affect appetite. Inulin is a fructan, a polymer of fructose with high solubility, with 2-60 fructose monomers and in the particular study the average degree of polymerization were about 10. Fructans with 10 sugar monomers or less are classified as fructooligosaccharides (Niness, 1999). Fructans are not available for our endogenous enzymes, but are readily fermented by the intestinal microflora. Short chain FOS in moderate doses (5 g and 8 g per day) can increase colonic fermentation in a dose response dependent manner within 240 minutes after a single serving (Hess *et al.*, 2011). Habitual consumption of 16 g FOS (Raftilose P95, 6.27 kJ/g) each day for two weeks reduced the energy intake during a 24 hour period compared to equal doses of dextrin maltose (Cani *et al.*, 2006). Inulin and fructans are also known to cause stomach irritation and two servings of 10 g FOS were found to have a positive correlation between rate of fermentation and flatulence and bloating, without any reduced appetite compared to a test product without addition of fiber (Karalus *et al.*, 2012). However, a single 16 g serving of Raftilose P95 did not cause such adverse effects (Peters *et al.*, 2009). DF fermentation in colon seems to possibly have some effect on appetite and energy intake; on the other hand the risk of causing stomach irritation has to be considered. It would be surprising if the adverse sensations in the digestive tract seen with fructans did not affect feelings of fullness, hunger or desire to eat. However, any event of colonic fermentation will most likely be reflected in the plasma metabolome.

The “Rye Factor”

Rye products may lower postprandial insulin response in both healthy persons and persons with type II diabetes (T2D) (Juntunen *et al.*, 2003; Rosen *et al.*, 2009; Breen *et al.*, 2013). Low postprandial insulin may in long term reduce risk of developing T2D (Ludwig, 2002). Reduced insulin response is also beneficial for patients with developed diabetes and it may also affect appetite. Lower postprandial plasma insulin relative to plasma glucose have repeatedly been observed with

rye diets compared to WWB references in healthy subjects (Leinonen *et al.*, 1999; Juntunen *et al.*, 2003). This phenomenon has been called the "rye factor" and acts through reducing the need of insulin for glucose regulation and has been proposed to be an effect of the structure of rye bread. One hypothesis is that the rye factor occurs due to reduced accessibility for amylases and thus, decreased rate of glucose absorption (Juntunen *et al.*, 2003). This may be due to formation of amylose that shields the starch for degradation and due to viscous dietary fiber. Another possible explanation is the lower amounts of branched chain amino acids observed in plasma after rye consumption (Moazzami *et al.*, 2012). These amino acids promotes non-glucose dependent postprandial insulin secretion and contribute to development of insulin resistance in rats (Newgard *et al.*, 2009). In diabetics, a single serving of pumpernickel bread (a dark German wholegrain rye bread baked up to 20 hours) lowered both postprandial blood glucose and insulin levels compared to a WWB (Breen *et al.*, 2013). This does not follow the idea of the rye factor, but demonstrates insulin lowering effect of rye in persons with developed T2D. In addition, these results were correlated with reduced appetite after pumpernickel consumption. The authors stress the importance of soluble fibers, as found in rye, but to a lesser extent in wheat, to cause the positive effect on blood glucose and insulin levels. Not only whole grain rye bread, but also rye bread made from endosperm flour can lower the postprandial insulin response and decrease appetite compared to a WWB control (Juntunen *et al.*, 2003; Rosen *et al.*, 2009). Rosén et al. (2009) propose that there may be some correlation between the insulin lowering effect of rye and reduced appetite.

Overweight and obesity are also important when studying satiation and satiety responses and it has been demonstrated that obese persons (BMI 33.7+/-0.7 kg/m^2) do not respond with a lower appetite after a wholegrain rye bread serving compared to a wholegrain wheat bread (Heinonen *et al.*, 2007). Levels of the meal initiating hormone ghrelin was unchanged in the obese persons after both breads, but in a group of lean persons ghrelin levels were, as to be expected, reduced postprandial with low fiber wheat bread. Interestingly, the obese persons exhibited a lower insulin response and reduction in circulating insulin over 120 minutes after the rye meal, but with no changes in the blood glucose compared to the WWB. This supports the idea of a rye factor that reduces the postprandial need for insulin to regulate blood glucose. The satiating effect of the rye bread may in this case be masked by distorted satiation and satiety systems in the obese research subjects. The insulin lowering properties of rye may nonetheless be clinically important, considering the correlation between obesity and T2D.

Proteins

Protein is known to have a higher satiating power compared to carbohydrates and fats. For instance, increasing protein in a single meal from 10 E% to 25 E% by replacing carbohydrates with higher amounts of animal protein can decrease appetite during a 4 hour postprandial period (Smeets *et al.*, 2008). Similar effects have been reported for soy protein (Veldhorst *et al.*, 2009). However, most research has investigated proteins of animal origin. Cereal proteins with their amino acid profile and protein composition differs from e.g. legumes and meat. Thus, they deserve special regard. Cereal proteins are mainly structural and functional with lysine as the limiting amino acid. In legumes proteins serves as energy storage generally with methionine as the limiting amino acid. Among cereal proteins, gluten of wheat is a protein complex widely studied for its functional properties in bread making, but less is known about the nutritional properties. The satiating effect of gluten did not differ from that of proteins from egg albumin, casein, gelatin, soy, and pea in a complete lunch serving, (Lang *et al.*, 1998). Despite about 13.8-15.5 E % of proteins were modulated in the study, this might not have been enough to see any effect. Noteworthy, the total energy content of 5.1 MJ in the test meal was considerably large compared to 8-10 MJ being the average daily intake for adults. Providing preloads of 50 g of gluten, soy or whey protein did not reveal differences in energy intake at an *ad libitum* lunch 2 hours after the preload (Bowen *et al.*, 2006). Compared to a glucose control, energy intake and postprandial insulin was lower for all three proteins. The satiating effect of both plant and animal proteins were higher compared to a carbohydrate source, and the satiating effect of gluten proteins seems to be at least equal to proteins from other sources.

In summary, rye seems capable of reducing the perceived appetite not only directly after consumption, but also in the period after consumption of the next meal, compared to low fiber wheat products. In addition, consumption of both low fiber and high fiber rye products has the capability of reducing insulin response after a meal. However, insulin response and appetite reduction are not the same in all cases of rye. Whole kernels promote satiety over milled rye and did so, only in a porridge matrix. However, endosperm rye bread has proved to be equal to a wholegrain counterpart regarding both appetite reduction and insulin lowering effect. Different growing sites and varieties may also introduce variables that possibly affect appetite. In obese persons the insulin lowering effect was found, but appetite reduction was absent (Heinonen *et al.*, 2007). To further investigate and make causal connections between rye and satiety a number of properties needs to be assessed, such as; physiological and chemical quality of the raw material, food matrix and processing and also a healthy satiation system of the test subjects. In addition serving size, caloric content and available carbohydrates has to be considered and adjusted for.

Metabolomics and chemometrics

Metabolomics refers to the quantitative and comprehensive study of metabolites in an organism (Dettmer & Hammock, 2004). It can be described as a snapshot of all small metabolites constituting an organism's phenotype in its tissues or fluids and is the latest of the "omics"- sciences, preceded by genomics, transcriptomics and proteomics. In humans, blood and urine samples are commonly the target for this holistic type of analysis. In contrast to the other "omics" metabolomics provides broader information of the phenotype, with endogenous metabolites reflecting both genetic setup and response, as well as exogenous metabolites from exposure to environmental factors. Dietary responses include all of the above through influences of e.g. the composition of the tested food, the genome and gut microflora of the person eating the food, and exposure to other environmental factors. Thus, analyzes at the metabolome level are useful in exploring and explaining responses and effects of diet in human subjects.

The untargeted metabolomics approach is primarily a tool generating new hypotheses and prediction models (Kell, 2004). The main hypothesis when using the untargeted approach is that there is systematic variation of metabolites, e.g. between treatment group and control or between baseline and later time points or between treatments. The aim when choosing analytical methods for metabolomics is to find a method that detect and quantitate as many metabolites as possible, rather than aiming at in-depth exploration of an *a priori* defined set of metabolites.
The two main analytical technologies for metabolomics analyses are Nuclear Magnetic Resonance (NMR) and chromatography methods coupled to mass spectrometry. The advantage with NMR is the ability to detect a wide range of molecules at an acceptable rate of speed. Modern NMR has also the advantages of high reproducibility and no need for separation or derivatization which is the case for mass spectrometric based metabolomics techniques (Malet-Martino & Holzgrabe, 2011).

Treatment of the vast amount of data produced in chemical composite analyses is handled within the field of chemometrics. Chemometrics uses mathematics and multivariate statistics to make descriptive and predictive models to sort out underlying patterns in complex datasets (Kumar *et al.*, 2014). Principal component analysis (PCA) is often one of the first measures in chemometrics to explore and visualize response patterns. PCA reduces the dimensionality of data unsupervisedly by seeking orthogonally oriented distributions, called principal components (PC), in a three dimensional data matrix, (Wold *et al.*, 1987). The PCs create a new set of variables in which each PC is representing the greatest uncorrelated variations in the original dataset. Plotting PCs against each other in two or three dimensions visualizes groupings and possible outliers in the dataset. This information can be used for further statistical measures. To investigate co-variance,

multivariate regression analysis such as Partial Least Squares (PLS) is used. Discriminant Analysis (DA) PLS is a variant used for classifying data by adding categorical response variables (Trygg *et al.*, 2007). To be able to use the feature that each subject act as their own control in crossover studies multi-level (ML) PLS-DA have recently been developed and can be viewed as multivariate paired t-tests (Westerhuis *et al.*, 2010).

Aim

The main aim of this thesis was to evaluate and develop an untargeted NMR-based metabolomics tool to assess effects of rye diet enriched with plant protein and fermentable fibers on appetite. The analyses were performed on plasma samples from healthy men and women (n=21) participating in short term controlled crossover breakfast study. The thesis also aims to investigate and summarize current knowledge on the relationship between rye-based products and appetite. The focus of this thesis was oriented towards the 55 g rye porridge treatment (without any supplement of plant protein and fermentable fiber) and the wheat control as they were supposed to best express the effect of rye on the plasma metabolome.

Material and methods

The experimental part of this thesis was a part in a larger intervention study performed in 2013 at the Department of Food Science at Swedish University of Agricultural Sciences (SLU) in collaboration with Uppsala University and the Swedish agriculture cooperative corporate group Lantmännen. The objective of the intervention was to investigate the effect of rye diets supplemented with plant protein and easily fermentable fiber on satiating mechanisms, particularly relationship between perceived appetite and changes in the metabolome and hormonal levels. The choice of rye matrix was based on its association with higher satiety compared to refined wheat bread. The addition of easily fermented fiber would provide information on how fermentation affects satiety, while the plant protein was added to increase insight to satiating mechanism of a specific plant protein. The results presented here are mainly focused towards the workflow of treatment of data and description of features of the plasma metabolome dataset. Plasma samples and statistical data on subjective appetite used in this thesis were obtained from the previous work within the larger study.

Subjects

Participants were recruited by advertisements in local newspaper and at SLU and after the initial selection and screening 21 healthy subjects were selected. The group consisted of 11 men and 10 women between 23-60 years of age (mean 38.6±11.8 years) with a BMI of 20.7-33.2 kg/m^2 (mean 24.9±3.3 kg/m^2). Habitual consumption of breakfast, lunch and dinner was required. Assessment of eating habits was performed, using three factor eating questionnaire to exclude cognitive restraint, emotional eating and uncontrolled eating. This was done to select persons with normal eating behavior suitable for a dietary intervention. Physical or psychological problems affecting eating together with the use of diets and tobacco were criteria for exclusion. Physically active persons with high physical activity level (PAL) scores (> 1.8) or fasting levels of glucose, thyroid-stimulating hor-

mone, hemoglobin and alanine aminotransferase deviating from normal values were also excluded. Attending pre-menopausal women were required to use hormonal contraceptives to control for fluctuating hormone levels that may influence appetite.

Study design

The study was designed as a single blinded, randomized cross-over study and was run over twelve weeks with six treatments. Each participant received one treatment at one day, followed by a six-day washout period. Before each visit to the study center at Uppsala University Hospital, Sweden, the subjects were asked to avoid dietary fiber and gelatin rich foods in their last meal and arrive fasted from 20.00 the day before. They were also told to avoid strenuous physical activity and alcohol during the last 24 hours before each visit. The test breakfast was served in the morning and effects were evaluated during 8 hours. At 240 minutes a standardized lunch was served and at 480 minutes an *ad libitum* dinner was served. Food consumption at dinner was registered and energy intake was calculated. Participants stayed in the study center all day doing sedentary activities.

Meals

The test meals were five rye porridges and a white wheat bread control (WWB). Two levels of rye was tested, 40 g (R) and 55 g (R55). Three levels of easily fermented fiber and plant protein were also tested with 40 grams of rye flour, RHP,

Table 1. *Characteristics of the iso-caloric servings of rye porridges and WWB control. All values are for the complete meal with milk, fat and jam.*

Treatment		Serving size (g)	Rye flour (g)	Added Easily fermented fiber (g)	Added Plant protein (g)	Energy (kJ)	Dietary fiber (g)	Carbohydrate (g)	Protein (g)	Fat (g)
Rye High Fiber	RHF	333	40	9	3	1372	10.0	46.3	19.6	5.0
Rye Prot./Fiber	RPF	334	40	6	6	1368	12.9	46.4	16.6	5.6
Rye High Protein	RHP	336	40	3	9	1363	15.9	46.4	13.6	6.1
Rye 40 g	R	332	40	-	-	1364	6.8	45.9	10.6	9.9
Rye 55 g	R55	384	40	-	-	1364	9.2	54.5	12.0	4.7
Wheat Bread	WWB	342	0	-	-	1369	2.1	47.4	11.6	9.8

RHF and RPF (Table 1). The porridges were prepared from steamed rye flakes soaked in boiling water for 4 minutes. To each serving 100 g of milk and 25 g of raspberry jam was provided to give a more palatable meal. Margarine was added to the porridge in various amounts to obtain iso-caloric servings. To WWB 150 ml water was served to adjust for the higher mass of the porridges.

A lunch of pork stew with rice (405 g servings, energy content; 597 kJ/100 g) was served 240 minutes after breakfast. To validate the satiating effect over 8 hours an *ad libitum* dinner consisting of a pasta Bolognese buffet was served at 480 minutes and energy intake was calculated. In addition a maximum of 100 ml water was allowed during the day and this intake was to be standardized for each of the following test days.

Blood sampling and appetite assessment

For metabolomics, venous blood samples were collected in lithium heparin tubes 15 minutes before breakfast and at 35, 65,185, 230, 305, 365 and 470 minutes after the breakfast. Collected samples were stored for several months at -80 °C until analysis. To asses subjective appetite, visual analogue scale (VAS) was used on a handheld Palm Pilot computers (Palm Inc., USA) (Blundell *et al.*, 2010). Participants estimated their perceived appetite every 30 minutes, beginning 30 minutes before breakfast and throughout the entire day with the last registration just before dinner 8 hours after breakfast. They answered three questions: “How full do you feel?”, “How hungry do you feel?”, and “How big is your desire to eat?”.

Sample preparation

To remove proteins that would prevent detection of smaller metabolites from plasma samples, an ultrafiltration protocol for serum was used (Tiziani *et al.*, 2008; Moazzami *et al.*, 2012). Ultrafiltration tubes (Nanosep 3k Omega, Pall Corporation, USA) were prepared by washing 6 times with 500 µl Millipore water and centrifuged at 2000 RCF at 36-40°C for 8 minutes. Filters were kept wet until application of samples within 48 hours. The plasma samples were thawed on ice and thoroughly vortexed at high speed. Plasma (500 µl) was pre-centrifuged at 12000 RCF and 4°C for 15 min to precipitate solids. Then, 400 µl plasma supernatant was applied to the filters and centrifuged at 12000 RCF and 4°C until sufficient elution (5-8 hrs). Filtrate was stored for several weeks at -80 °C until NMR analysis.

NMR samples were prepared by mixing 250 µl deproteinized plasma, 125 µl Millipore water, 150 µl phosphate buffer (0.4 M, pH 7.0), 45 µl D2O (99.96 % D atom), and 30 µl trimethylsilyl propanoic acid (TSP) (5.8 mM in D2O). Samples

(560 µl) were analyzed in 5 mm NMR tubes on a Bruker spectrometer at 600 MHz.

Pre-treatment of NMR data

Trimethylsilyl propionic acid (TSP) standard peak was manually adjusted to zero, phase corrected and baseline corrected in TopSpin™ 2.0 (Bruker Corporation, USA) software. The adjusted spectra were imported to the software programming language R (v. 3.1.0) using "readBruker" function from the Bayesian Automated Metabolite Analyser for NMR spectra (BATMAN) package. To detect peaks in NMR spectra and to compensate for small differences in shift between the peaks in different samples, the speaq 1.1 (Spectral Alignment and Quantification) package in R was used to align all spectra (Vu *et al.*, 2011). The speaq package provides automated peak finding and alignment of spectral datasets based on hierarchical Cluster-based Peak Alignment (CluPA). Imported spectra was integrated over -0.05 – 0.05 ppm and normalized to 0.25 mmol/L TSP concentration. Peak detection was performed using the "detectSpecPeaks" function (nDivrange = 256, scales = seq(1,16,2), baselineTresh = 0.05). Reference spectrum was determined using the "findRef" function. All spectra were aligned to the reference with the "dohCluster" function allowing a shift adjustment of maximum 100 points. The "dohCluster" function uses hierarchical clustering to track peaks in the target spectrum peaks against a reference spectrum by changing shift of peaks in target spectrum. The reference spectrum does not contain all peaks that possibly are present in the dataset, but is rather like an average representative of the whole dataset. In a complex dataset, for example plasma samples from different individuals and time points, some metabolites are only expected to be present in some of the samples. These metabolites are of equal interest and need to be added to the final dataset through the creation of a new reference vector by adding peaks from all other spectra to the reference spectrum. This was done by subjecting the aligned spectra to a second peak picking, set to a more generous scales setting (scales = seq(1,8,1)), allowing more peaks to be detected. Peaks identified in the aligned spectra were added to the final peak list.

In two regions, 7.94-7.89 ppm and 7.12-7.08 ppm, the alignment algorithm was not sufficient to align two apparent peaks. All identified peaks were removed from these regions and substituted with the local maxima of each spectra subsequently used for alignment. The regions 5.4-6.2 ppm and 7.5-7.8 ppm were dominated by one broad peak each. The peaks were well aligned, but a lot of additional signals were detected. To reduce the noise, all peaks in these regions were removed and substituted with local maximum.

Statistical analyses and identification of metabolites

Appetite ratings were analyzed with a repeated measures mixed model with least square means in SAS version 9.3 (SAS institute Inc., USA). Data visualization and statistical analyses of the blood plasma NMR spectra were performed in The Unscrambler® X 10.1 (CAMO Software AS, Norway)using Principle component analysis (PCA) and plotting of scores and loadings. Partial least squares discriminant analysis (PLS-DA) validated with Non-linear Iterative Partial Least Squares algorithm (NIPALS) was used to discriminate response classes. To identify metabolites, 1H NMR spectra and shift tables from Human Metabolite Database were used together with a spectra profile of 48 serum metabolites identified using Chenomx NMR suite (Chenmox Inc., Canada). The Chenomx suite use NMR library data on specific compounds that can be adjusted to fit desired NMR conditions such as pH and ionic strength. Reference NMR profiles were built from the adjusted reference compounds, and peak identification and quantification in sample spectra were performed by manual adjustment of each peak. A general down shift of the spectra of about 0.019 ppm was used when compared to HMDB data. Tolerance for identification with the database shift tables was +/- 0.005 ppm.

Results

A single breakfast of rye porridge was found to reduce feeling of hunger compared to the iso-caloric WWB breakfast over an 8 hour period. This reduced hunger compared to WWB was found in four of the treatments; R55 ($p = 0.0001$), RHF ($p = 0.0005$), RPF ($p = 0.0015$) and RHP ($p = 0.0482$), regarding the question "How hungry do you feel?". Rated hunger with Rye 40 g did not differ from WWB ($p > 0.05$). No differences for any of the treatments were measured with "How full do you feel?" and "How big is your desire to eat?" when looking at the full 8 hour period, as variation between single time points were interfering with the overall trend.

Peak picking and alignment

The speaq-package with automated peak detection and alignment algorithms detected in total 1231 peaks in NMR spectra from deproteinized plasma samples of the 21 individuals, the six treatments and eight time points ($n = 994$ samples). However, a majority of these peaks were found to be noise. Visual inspection of NMR spectra and identified data points showed several regions where many peaks were in the range of baseline noise. Figure 1 show one such region around 2.8 ppm where many identified data points do not represent actual peaks. Around 4.5 ppm the reference spectrum appeared to have a broadened baseline, suggesting that this spectrum may not be the representative (Figure 1). These regions contributed with a considerable amount of noise, but were kept in the dataset to test if such noise reduced interpretability of the statistical model or not. It was also obvious that the peak picking model was not able to detect all peaks that were present; e.g. between 3.45-3.50 ppm three peaks were not included in the reference spectrum although they obviously were above baseline noise (Figure 2). These peaks were not detected in the second peak picking although settings allowing for detection of more peaks were applied. A manual screening of a subset of 100 spectra resulted in 98 peaks that were not recognized by the peak picking algorithm and thus, not includ-

ed in the subsequent multivariate models (Data not shown). When comparing subsets of spectra before and after alignment there was noticeable improvement in spectra pattern fit. However, several spectra showed shift of peaks to wrong frequencies.

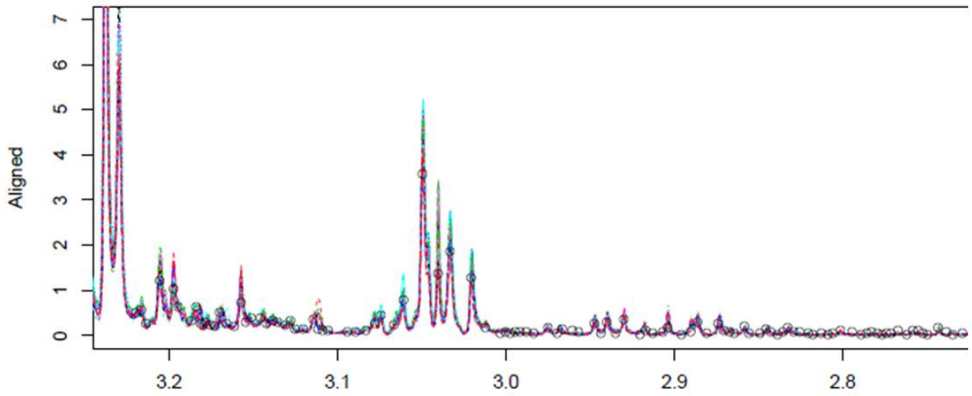

Figure 1. Plot of 20 aligned plasma NMR spectra 3.25-2.75 ppm showing "peaks" at 2.8 ppm from reference spectrum that are embedded in the baseline noise. Circles represent identified peaks and peak heights from the reference spectrum.

Normalization of spectra was performed as a trapezoid function of the peak area of TSP. In the peak list used for the multivariate analyses, peak heights were used for peak quantifications. Given that shimming conditions and impurities in the sample affects the width and height ratio of NMR peaks, peak height is not an optimum measure of signal intensity and may vary with NMR conditions. For all samples in the set the average TSP peak height was 124.8 (SD = 14.4, SEM = 0.47) and regarded as acceptable.

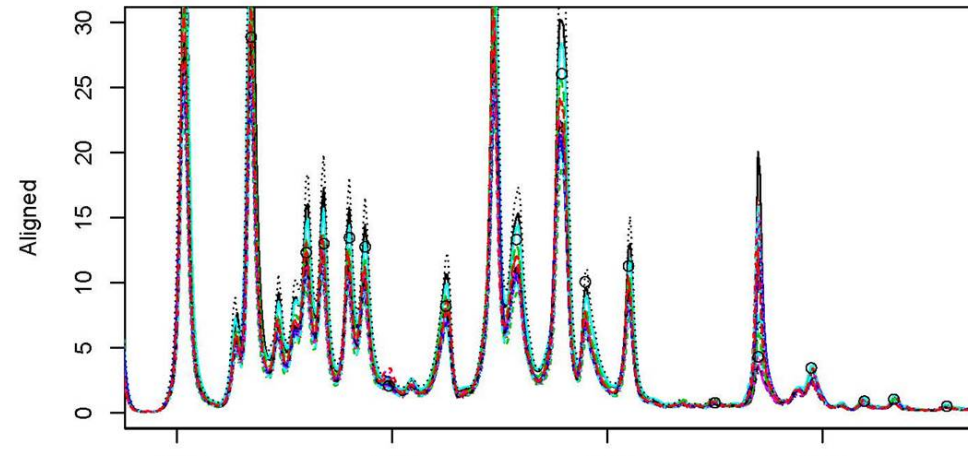

Figure 2. Plot of 20 aligned plasma NMR spectra showing three true peaks at ~3.48 ppm that are not represented in the reference spectrum. Circles represent identified peaks and peak heights from the reference spectrum.

Multivariate analyses

No differences between the R55 breakfast and WWB were detected with the applied PLS-DA method in the whole study sample or even in single individuals. Nevertheless, useful information on data treatment and important features of the plasma metabolome dataset from the dietary intervention were obtained.

PCA analysis on the full data set revealed no differences or even tendencies of separation of samples according to treatment, individual or time point (Data not shown). Loadings were largely explained by TSP and the two larger lactate peaks at 1.33 ppm. All identified metabolites and their detected shifts are noted in Appendix 1. These two metabolites were excluded from the following PCA analysis to see if metabolites that better explained treatment variation emerged. Also, glycerol was excluded due to contamination by glycerol from the filter used for deproteinization. In the PCA model without TSP, lactate, and glycerol neither treatment nor time points could be separated by the first two components, but a pattern of time point groups were emerging (Figure 3). Loadings of PC1, explaining 60 % of variation was dominated by glucose peaks in the 3.2-3.9 ppm region and PC2 (10 %) was explained by lactate at ~4.12 ppm and alanine at ~1.46 ppm (Figure 3). To examine the involvement of other metabolites into treatment dependent variation, lactate and glucose were excluded from the model. To exclude glucose, all data points in the complete region 3.22-3.92 ppm, possibly containing 44 glucose peaks, was removed. The two doublets at 5.2 ppm and 4.6 ppm of glucose were not emerging on the loading plot and were kept in the set. In this analysis, the dataset was limited to the R55 and WWB treatments, which expressed significant differences in appetite ratings and were expected to differ on the basis of metabolome.

In the R55/WWB PCA model less variance was explained by two first components. PC1 (24 %) was mainly influenced by alanine peaks at 1.48 ppm and in PC2 (20 %) an unidentified peak at 3.049 ppm was dominating (Figure 4).

Scores plot of the R55/WWB PCA model showed separation over PC2, but there was no grouping by treatments and grouping by time points seemed less evident than in the PCA model which included all treatments and glucose (Data not shown). When 8 random individuals were highlighted in the scores plot it was evident that between persons variation, especially over PC2, was an important explanatory factor (Figure 5).

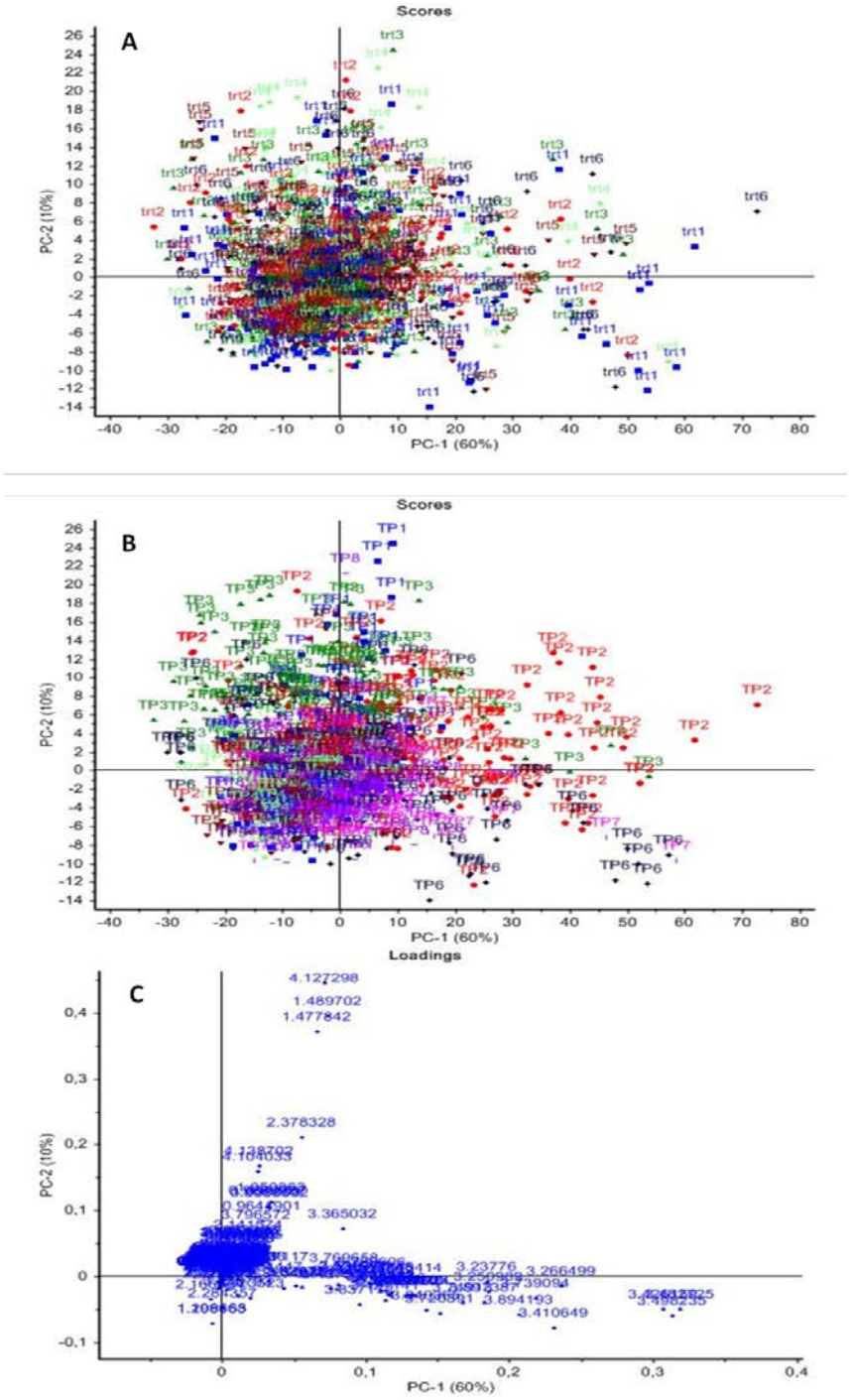

Figure 3. PCA plots of complete dataset. **A** – Scores plot with treatments indicating no systematic effects of treatment. **B**- Scores plot with time points indicating some time point dependent effect, especially over PC1. **C**- Loadings plot with x-loadings (Specific NMR shifts of detected peaks), contributing to PC1 and PC2. PC1 is characterized by a glucose peak cluster at 3.2-3.9 ppm and PC2 by two alanine peaks at 1.48 and a lactate peak at 4.12 ppm.

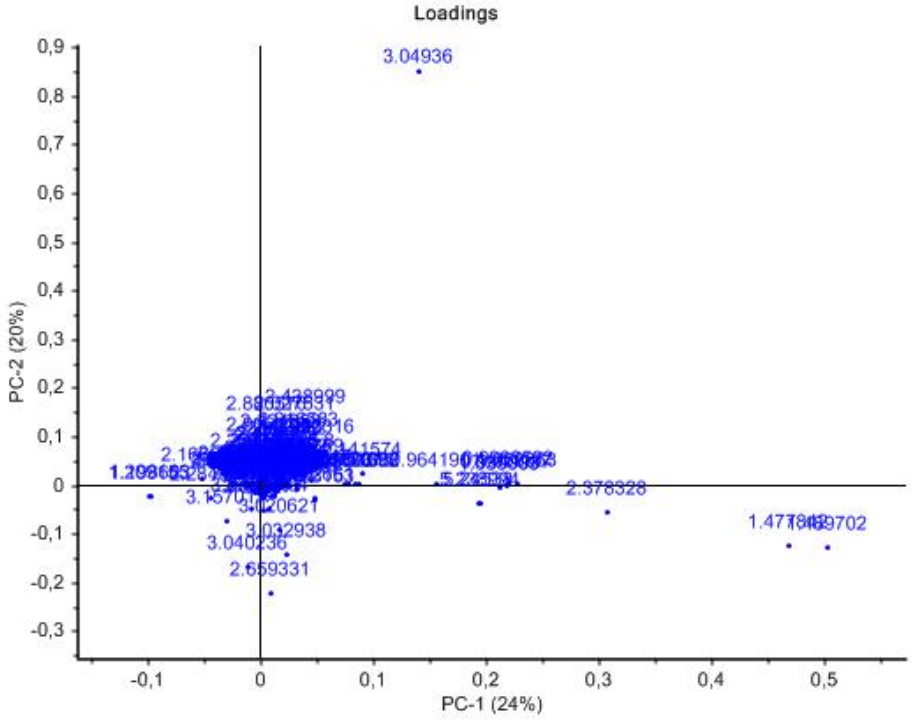

Figure 4. PCA x-loadings of a subset with R55 and WWB peaks for all individuals. Lactate, TSP, glucose and glycerol were excluded from the model. The alanine doublet peaks at ~1.48 ppm to the right in the plot characterize PC1 and PC2 is mainly described by a single signal at 3.049 ppm.

The complexity of the model was still quite high with 1147 peaks, of which a lot was identified as noise when visually examining spectra plots. There were 3 main regions, 0.06-0.82 ppm, 4.3-6.9 ppm, and 7.47-10 ppm, which contained many identified features that were within the range of baseline noise (Appendix 2). Data from these regions were manually removed from the set, with exception for peaks that in several samples were deviating from the pattern of the baseline noise. The reduced dataset, also without the previously removed TSP, glycerol, glucose and lactate, contained 585 data points for each sample. When this noise reduced R55/WWB set was subjected to PCA, the explained variance did not increase and no new clustering was observed. To possibly overcome the observed variance between individuals and between time points, a discriminant analysis was performed on the R55/WWB set with R55 as the class variable tested against WWB. This model could not discriminate the R55 metabolome from WWB metabolome.

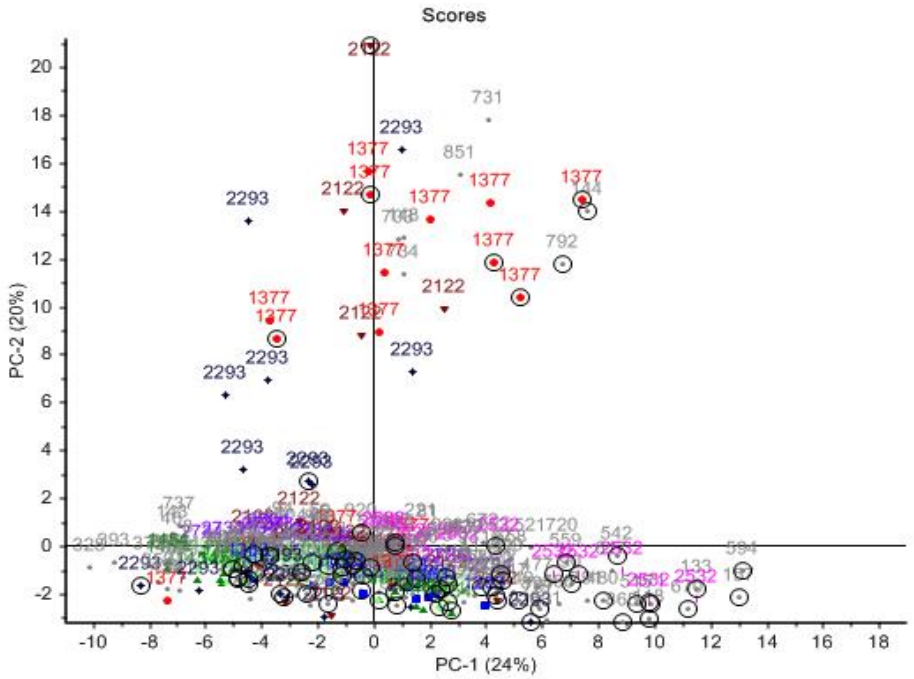

Figure 5. PCA scores for all individuals the in R55 and WWB subset showing contribution to PC2 by some individuals. Highlighted individuals are labeled with four digit numbers. Circles denote outliers.

To remove between-individual variations, PLS-DA was performed on individuals using the noise reduced R55 and WWB dataset (Figure 6, Table 2). Time constraints and the prospect of being able to try more different models limited the studied individuals to four randomly selected datasets. Prediction of all models was good with r^2 ranging from 0.900 to 0.985, but r^2 of the validation model was very low for ID 1377 ($r^2 = 0.292$) and ID 1701 ($r^2 = 0.400$) (Table 2).

Table 2. *Fit of predictive and validation PLS-DA models of R55 vs WWB in single individuals. Left column shows fit with all metabolites in the noise reduced set. Right column shows fit without those explanatory variables that were most influential in the model with all metabolites.*

ID	All metabolites			Without metabolites having the largest contribution to the first model		
	No. of factors explaining model	Prediction r^2	Validation r^2	No. of factors explaining model	Prediction r^2	Validation r^2
1377	5	0.933	0.292	5	0.986	0.856
1454	4	0.900	0.695	4	0.919	0.737
1701	7	0.985	0.400	5	0.996	0.894
2434	6	0.982	0.783	5	0.994	0.908

To improve the validity of the PLS-DA model, peaks with the highest contribution to the first model were removed from the dataset. This operation was done to remove peaks that contributed to prediction of class, but that did not describe the model very well. The new PLS-DA model increased both the prediction r^2 and validation r^2 for all individuals (Table 2). In ID 1377, for example, the increase of r^2 of the validation model was largest, from 0.292 to 0.856. Score plots showed slight separation of R55 and WWB over factor 1 and 2 for all individuals (Figure 6). To investigate if this separation was caused by the treatment or by other factors, time points scores were plotted and connected with trajectory lines to see the direction of class separation in each time point (Figure 6). Most of these trajectories point in the same direction. This was also true for baseline samples drawn before the test breakfast (TP1) except in the case of ID 1701 that describe a different direction for TP1. The similar direction of TP1 and all time points after treatment suggests that the initial metabolome is more important in the differences between two groups, rather than the treatment. Examination of the peaks and metabolites contributing to discrimination of R55 and WWB treatments indicated that there were different metabolites that contributed in each individual and most of them were not identified. However, in ID 1701 valine peaks (0.987 ppm, 1.039 ppm, and 1.051 ppm) were separated from R55 loading (Data not shown).

With the described discriminant models no treatment effect between the R55 and WWB treatments was found. Instead, time point, between individual variation and history of food eaten by the studied persons before each occasion seemed to describe the observed variation better.

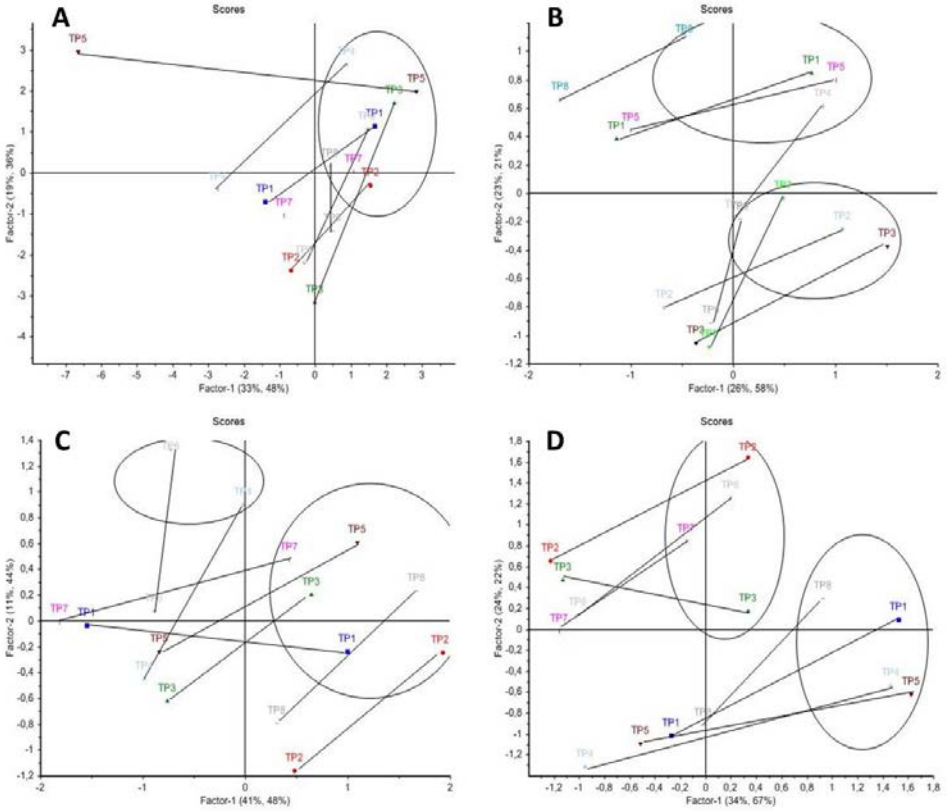

Figure 6. PLS-DA scores plots of R55 and WWB discrimination with time points labeling. **A** – ID 1377, **B** – ID 1454, **C** – ID 1701, and **D** – ID 2434. R55 treatments are enclosed in the circles and WWB are outside. TP1 = -15 min, TP2 = 35 min, TP3 = 65 min, TP4 = 185 min, TP5 = 230 min, TP6 = 305 min, TP7 = 365 min, TP 8 = 480 min.

Discussion

The decreased feeling of hunger with the R55 rye porridge breakfast compared to WWB is in agreement with the previous findings (Isaksson *et al.*, 2008, 2011). However, whether this effect was reflected in the plasma metabolome over 8 hours could not be clarified. This may be explained by the high dimensionality in the study and is also probably a result of the small effects a single breakfast may have on the metabolome. Moreover, the differences in satiety were in the magnitude of 20 % between the treatments.

In the PCA score plot with the whole dataset, the clustering of time points was explained by differences in glucose over time after the meal, which could be anticipated. Glucose contributes to the plasma NMR profile not only by high concentration in plasma compared to most other metabolites, but also by contributing with 48 peaks. In the used PCA model, all peaks are weighted equally. This means that although spectra were normalized, the relative contribution of glucose to the total variation would be higher and may shade explanatory metabolites with fever peaks. It should be noted that the strategy to remove glucose was not self-evident, since rye can lower blood glucose concentrations in comparison to WWB, and thus, an important identifier of a treatment effect may have been neglected (Breen *et al.*, 2013). Lactate plays a vital role in the energy metabolism and is changing with substrate utilization and oxygen supply in the cells (Kreisberg, 1980). The large influence of lactate in the PCA model was most likely not contributing to explanation of any possible treatment effect, but rather reflected differences between individuals.

The negative correlation of valine to R55 treatment in one person is a small clue worth noticing, since lower levels of plasma branched chain amino acids have been found in rye bread compared to WWB (Moazzami *et al.*, 2012). Those findings were made with a targeted approach that has a more easily interpreted model. A targeted approach may also be used on the data from the present study to try to find satiety related differences in the metabolome between the rye treatments and

WWB. The drawback with such an approach would be that candidate metabolites differing between treatments are limited those included in the analysis from *a priori* generated hypotheses.

The observed time point dependent tendencies are by themselves another source of variation that has to be dealt with in the statistical models. The variation of metabolite concentrations in the blood between starvation and feeding periods are fluctuating as anabolic and catabolic pathways are altered. One way to deal with such variation could be to look at each time point separately. This might bring forward metabolites that can be connected to treatments. On the other hand, this will decrease statistical power of the models since the number of samples dramatically is reduced.

Different individual are also expressing different metabolome due to genes and life style. Adult females for example, have been associated with higher concentrations of several lipid metabolism related compounds, while men were characterized by more amino acid metabolism compounds when using NMR based metabolomics (Kochhar *et al.*, 2006). Another example is older people, whose metabolomes have been described by metabolites related to increased muscle degradation (Lawton *et al.*, 2008). Even with a standardized diet over two weeks, a group of people (n = 12) exhibited large between person variation in the plasma metabolome (Lenz *et al.*, 2003). Diets rich in phytochemicals have also proved to influence the plasma metabolome with a profile rich in phenols and gut fermentation related hippurate (Walsh *et al.*, 2007). Foods eaten by the persons in the present study the days before the intervention had, most likely, a great impact on the metabolome, despite that they were fasted over-night prior to the test breakfast. Other lifestyle related parts of the metabolome will also be altered between each treatment day. MLPLS-DA may have the potential to resolve at least the between individual variation (Westerhuis *et al.*, 2010). This fairly new variant of PLS-DA was used by Moazzami *et al.* (2012) in a targeted NMR metabolomics study of rye bread and isoleucine, leucine, betaine and N,N-dimethylglycine were found to be the major contributors to difference from WWB. Absolute concentrations for those metabolites after selection in the MLPLS-DA model were subjected to a paired t-test and all four metabolites were different between rye and WWB treatments. Moazzami *et al.* (2012) were studying fasting samples after an 8 week intervention, while the present study was analyzing postprandial samples after a single serving. Still, valine was found to contribute to discrimination of R55 and WWB in one of four randomly selected individuals. With improved statistical models, resolving the issue of between-person variation, branched chain amino acids may emerge as contributors to a short term metabolome of rye diet as well. The flux in the plasma metabolome and the “snapshot” nature of a metabolome sample is the

advantage of metabolomics, but also a difficult obstacle to overcome when trying to elucidate effects caused by treatments.

Data pretreatment is another crucial step in metabolomics studies. In the present study the "speaq" package was used to align and reduce the NMR dataset only to data points representing actual peaks. Vu *et al.,* (2011) stressed the importance of choosing an appropriate peak detection method to get a good alignment. It should also be emphasized that choice of peak detection method is crucial to maximize both sensitivity and specificity of the final analysis. In the present study the "detectSpecPeaks" function was used and complemented with a second peak detection that added peaks from all spectra to the final dataset. Many "true" peaks (i.e. peaks that were discernible by visual inspection, but not by the peak detection algorithm) were not included. This left out a lot of information that could have contributed to the explanatory models. Simultaneously, a vast amount of obvious noise was present within the identified features, which restricted the visual interpretation of data and possibly also interfered with the statistical models. These artifacts remained through intense testing of peak detection parameters. There were also some indications that the algorithm for reference spectrum identification did not performed excellently, since it may choose spectra with high number of identified peaks at the expense of signal-to-noise ratio. In addition, Vu *et al.* (2011) mentioned that high peak number is favored in this method and that it may be necessary to choose a lower rank spectrum. A manual inspection of the identified reference spectrum is a step in the workflow that should not be overlooked. The peak detection algorithm used in the present study may not be optimal and it can be necessary to use different methods in different regions of the spectrum (Vu *et al.,* 2011). A validation of the "speaq"-workflow is also needed and for that purpose the quality control (QC) samples that were included in the present study may be of excellent use. Due to time constraint, this step was overlooked in the present study. These QC samples could well reveal if the current peak detection and alignment is reliable and reproducible for this type of NMR data.

The quality of the in-data to the automated spectra treatment and the subsequent statistical modeling is of course crucial to improve interpretability of the results. In the present study several spectra suffered from baseline distortions, vertical shifts and, poor water suppression. They were nonetheless included in the peak picking and alignment model. Higher demands on the quality of the raw NMR spectra may improve the results.

The large contribution of TSP to the overall sample variation may look a bit odd but were originating from the fact that TSP was not normalized to peak height, but to peak area. The peak shape is dependent on NMR conditions and if TSP peak shape is biased in one way, it can be assumed that the rest of the spectrum is biased in the same way. Thus, the TSP peaks can contribute to the multivariate mod-

el with information that explains NMR condition dependent variation in the rest of the spectrum. The large contribution of TSP could also reflect an issue of weighting in the PCA model that may explain why strong signals such as glucose, lactose, and TSP had such strong influence on the first PCA models.

Conclusions

In this study several important steps towards an automated process to extract information from plasma NMR data were made. An automation of the data treatment is of vital importance for untargeted metabolomics, as current methods requires time consuming manual work, which sometimes is subject of arbitrary judgments. In the present study a large amount of features available for multivariate analyses were extracted within a very limited time. These data were extracted without any other criteria than what could be detected in the NMR and what signals that was possible to catch with the current algorithm, selectively, but untargeted.

The automated peak picking and alignment method included a lot of noise and left at the same time out valuable data. Further refinement and validation of the method is needed. It is also important to carefully choose statistical models that can handle the many biasing variables that had large impact on the total variation. Plasma metabolome could not discriminate between any of the six breakfast tested with the current method. Hopefully, with the following suggestions, refinement and interpretability of the plasma metabolome will increase so that small effects and fluctuations in the metabolome could be distinguished.

Recommendation for future research

For the continuation of this research project the following suggestions are made:

- Development of a new peak picking method or refinement of the current to increase sensitivity and specificity
- Scrutinize the normalization algorithm and its effect on final data
- Validation of the model for the speaq-workflow
- Development of a detailed workflow for how to assess sufficient quality of the raw NMR spectra
- Development of statistical models that handle the uncorrelated variation

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Appendix 1 - Shifts of identified metabolites.

Metabolite	Identified peak shifts (ppm)
Lactate	1.327, 1.339, 4.104, 4.115, 4.127, 4.139
Glucose	3.238, 3.251, 3.267, 3.411, 3.426, 3.483, 3.498, 3.720, 3.739, 3.749, 3.897, 5.239, 5.245
Alanine	1.478, 1.49
Acetate	1.924
Isoprop	1.173, 1.183
Valine	0.987, 1.039, 1.051

Appendix 2 - Removed NMR regions

Removed NMR regions containing detected peaks in range of baseline noise

Removed regions (ppm)
0.060 - 0.090
0.105 - 0.135
0.150 - 0.160
0.175 - 0.825
4.298 - 4.330
4.372 - 4.440
4.530 - 4.640
4.664 - 4.700
5.120 - 5.180
5.388 - 6.135
6.160 - 6.516
6.530 - 6.890
7.265 - 7.312
7.470 - 7.682
7.688 - 7.870
7.888 - 7.908
7.912 - 8.196
8.202 - 8.216
8.222 - 8.272
8.278 - 8.348
8.354 - 8.452
8.464 - 8.534
8.540 - 8.602
8.610 - 10.000

Appendix 3 – Popular summary

Rye products have in several studies been shown to contribute to greater satiety compared to white wheat bread. This study has shown that a breakfast consisting mainly of rye porridge served at one point gives a lower perceived feeling of hunger during the subsequent 8-hour period in 21 healthy and normal weight people . As well a porridge with 55 g of wholegrain rye as a porridge of 40 g of wholegrain rye with the addition of three levels of easily fermented dietary fiber and plant protein (9 g fiber / 3 g protein, 6 g fiber / 6 g of protein or 3 g fiber / 9 g protein) showed this difference in satiety compared to white wheat bread with the same energy content. Rye porridge of 40 g wholegrain rye was not different from white bread in perceived hunger and the applied statistical models were unable to distinguish the different breakfasts with respect to the satiety or the willingness to eat perceived by persons in the study. These results are in line with previous experiments, and the main question in this study is: Is the difference in hunger between the rye products and the white wheat bread reflected in the composition of small molecules, called metabolites, in the blood? The possible effects up to 8 hours after breakfast were considered.

Metabolomes is the whole set of metabolites in an organism. Larger molecules such as enzymes, hormones, structural proteins and complex carbohydrates are not normally included in the metabolome. For practical reasons only a part of the metabolome is in studied humans, ususally in the form of blood, urine, saliva or adipose tissue. Blood is the most frequently analyzed, since it is relatively easy to take such samples and blood quickly responds to different treatments. In addition, blood contains compounds that have been taken up by the intestines from the food and are to be transported to the parts of the body where they are needed, or to urine to be excreted from the body. The metabolome are comparable with the genome, which is an individual's entire set of genes. The difference is that while the genome is relatively stable and looks the same throughout life, the metabolome is constantly changing. These changes are due in part to supplying of substances to the body, primarily through the food, and in part to the body´s own regulation of the metabolome to maintain a balance that is required for us to live. This would allow the blood metabolome to be used to see if a person has eaten rye or wheat. And also, find out which substances in the diet that affect phenomena such as the feeling of satiety or hunger.

Metabolomics is the research field that studies the composition and changes of the metabolome in an organism. By measuring the presence of metabolites at one or more, both genetic and environmental factors contributing to the metabolome are reflected. The idea is to with a single sample see both metabolites of a compo-

nent of the diet, whilst at the same time be able to see the body's own reactions to the component. To provide as complete a picture as possible of these complex relationships, detection of as many metabolites as possible in every sample are desirable. In this study, blood samples from time points throughout the day were analyzed by Nuclear Magnetic Resonance (NMR) to obtain information on the atomic level on which substances are and how they vary. Primarily between the different breakfasts, but also variation between individuals, times during the day, and between the different trial sessions. This type of analysis provides a large amount of data for each sample, and combined with the six different breakfasts and the eight time points studied for each breakfast this aggregate many millions of data points for the entire experiment.

To get manageable amounts of data and still be able to retain as much useful information as possible, the software " speaq " (Spectral Alignment and Quantification) were used and adapted in this study. " Speaq" aims at, following given parameters, select and adapt the NMR signals to a format that makes it possible to identify and measure the quantity of various metabolites in blood samples. From the original data set from the NMR analysis a little more than 1,200 signals were sorted out. Of these, most were noise, i.e. redundant non-relevant information, and also many signals that corresponded to actual metabolites in the blood samples were omitted. Despite this, the result is considered positive when the data processing only took a couple of weeks, while the information may be sufficient to distinguish effects of the diet. To gather equivalent information with the methods commonly used today would take several months.

The statistical treatment of the data from the blood samples revealed no differences between the different types of rye porridge and the white wheat bread. For that reason, it was not possible to link the difference in perceived satiety to the metabolome. The indications found were that the metabolome clearly was linked to the increase in blood glucose that comes after a meal with carbohydrates. Also, the effects of what people had eaten the day before and the status of their body had at each test meal, seemed to have a large effect on the metabolome. Individuals also showed up metabolome which in some respects were different from others.

This study found no differences in the composition of small molecules in the blood that could be linked to the rye diet. This does not mean that the relationship should be dismissed. For example it has in previous studies been shown that rye diet produces a change in blood amino acid composition. More careful and developed statistics can hopefully handle the large differences that came by differences between individuals, between different test times and between different times of day. When that variance is under control it is likely that the underlying and less obvious differences in the blood may be linked to both rye diet itself and the increased feeling of satiety that comes with such a diet.

Appendix 4 – Populärvetenskaplig sammanfattning

Rågprodukter har i flera studier visat sig kunna bidra till en högre mättnadskänsla jämfört med vitt bröd. Den här studien har visat att en frukost bestående huvudsakligen av råggröt serverad vid ett tillfälle ger en lägre upplevd känsla av hunger under den efterföljande 8-timmars perioden hos 21 friska och normalviktiga människor. Såväl en gröt med 55 g fullkornsråg som gröt av 40 g fullkornsråg med tillsats av tre olika nivåer av lättförjästa kostfibrer och växtprotein (9 g fiber/3 g protein, 6 g fiber/6 g protein eller 3 g fiber/9 g protein) visade på denna skillnad i mättnad jämfört med vitt bröd med samma energiinnehåll. Råggröt av 40 g råg skiljde sig inte från vitt bröd i hungerkänsla och de tillämpade statistiska modellerna klarade inte av att skilja de olika fruostarna åt med avseende på den känsla av mättnad eller vilja att äta som personerna i försöket uppgav. De här resultaten är i linje med tidigare försök och huvudfrågan i den här studien är om skillnaden i hunger mellan rågprodukterna och det vita brödet kan återspeglas i sammansättningen av små molekyler, s.k. metaboliter, i blodet i upp till 8 timmar efter frukosten?

Metabolom är hela uppsättningen av metaboliter i en organism. Större molekyler som till exempel enzymer, hormoner, strukturella proteiner och sammansatta kolhydrater räknas normalt inte till metabolomet. Av praktiska skäl studeras bara delar av metabolomet i människor i form av blod, urin, saliv eller fettvävnad. Blod är det som oftast analyseras, eftersom det är relativt lätt att ta sådana prover och blod ger snabbt utslag på olika behandlingar. Blodet innehåller också de metaboliter som har tagits upp av tarmarna från maten som ska transporteras till de delar i kroppen där de behövs eller till urinen för att föras ut från kroppen. Metabolomet kan jämföras med genomet, som är en individs hela uppsättning av gener. Skillnaden är att genomet är relativt stabilt och ser likadant ut genom hela livet medan metabolomet är i ständig förändring. Dessa förändringar beror dels på att vi tillför ämnen till blodet, främst genom maten, dels på att kroppen hela tiden reglerar metabolomet för att upprätthålla den balans som krävs för att vi ska kunna leva. På det viset skulle blodmetabolomet kunna användas för att se om en person har ätit råg eller vete och även reda ut vilka ämnen i den kosten som påverkar fenomen som till exempel känslan av mättnad och hunger.

Metabolomik är det forskningsfält som studerar sammansättningen och förändringar av metabolomet i en organism. Genom att mäta förekomsten av metaboliter vid en eller flera tidpunkter återspeglas både gener och miljöfaktorers bidrag till metabolomet. Idén är att med ett prov både kunna se metaboliter från exempelvis en komponent i kosten och samtidigt se kroppens egna reaktioner på den komponenten. För att ge en så komplett bild som möjligt av dessa komplexa samman-

hang så försöker man upptäcka så många metaboliter som möjligt vid varje analys. I den här studien har blodprov från tidpunkter över hela dagen analyserats med kärnmagnetisk resonans (NMR) för att få information på atomnivå om vilka ämnen som finns och hur de varierar. I första hand mellan de olika frukostarna, men även variation mellan individer, tidpunkter under dagen och mellan de olika försökstillfällena. Den här typen av analys ger en stor mängd data för varje prov och sammantaget med de sex olika frukostarna och de åtta tidpunkter som har studerats för varje frukost ger det sammantaget många miljoner datapunkter för hela försöket.

För att få hanterbara datamängder och ändå kunna behålla så mycket användbar information som möjligt användes och anpassades i studien mjukvaran ”speaq” (Spectral Alignment and Quantification). ”speaq” syftar till att efter givna parametrar välja ut och anpassa NMR-signaler till format som gör det möjligt att identifiera och mäta mängden av olika metaboliter i blodproven. Ur den ursprungliga datamängden från NMR-analysen sorterades lite drygt 1200 signaler ut. Av dessa var den största delen brus, d.v.s. överflödig biinformation, och även många signaler som motsvarade faktiska metaboliter i blodproven var utelämnade. Trots detta kan resultatet anses vara positivt då databehandlingen bara tog ett par veckor, samtidigt som informationen kan vara tillräcklig för att skilja kosteffekterna åt. Att samla likvärdig information med de metoder som vanligtvis används idag skulle ta flera månader.

Den statistiska behandlingen av data från blodproven visade inte på några skillnader mellan de olika typerna av råggröt och det vita brödet. Av den anledningen gick det inte heller att koppla skillnaden i upplevd mättnad till metabolomet. De indikationer som fanns var att metabolomet tydligast var sammankopplat med ökningen av blodglukos som kommer efter en måltid med kolhydrater. Även effekter av vad personerna hade ätit dagarna innan och vilken status deras kropp hade vid varje testmåltid verkade ha stor effekt på metabolomet. Enskilda individer visade också upp metabolom som i vissa avseenden skiljde sig från andra.

Att den här studien inte fann några skillnader i sammansättningen av små molekyler i blodet som kunde kopplas rågkosten betyder inte att det sambandet ska avfärdas. Det har till exempel vid tidigare studier visats att rågkost ger en förändring i blodets aminosyrasammansättning. Mer noggrann och utstuderad statistik kan förhoppningsvis hantera de stora skillnader som kom av skillnader mellan personer, mellan olika testtillfällen och olika tidpunkter på dagen. När den variationen är fastställd så är det mycket möjligt att underliggande och mindre uppenbara skillnader i blodet kan kopplas till både rågkost i sig och den ökade känslan av mättnad som kommer med en sådan kost.