Research Article

NMR-based metabonomics reveals that plasma betaine increases upon intake of high-fiber rye buns in hypercholesterolemic pigs

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This study presents an NMR-based metabonomic approach to explore the overall endogenous biochemical effects of a rye *versus* wheat-based fiber-rich diet in hypercholesterolemic pigs. The pigs were fed high-fat, high-cholesterol rye- ($n = 9$) or wheat- ($n = 8$) based buns with similar levels of dietary fiber for 9 – 10 wk. Fasting plasma samples were collected 2 days before and after 8 and 12 days on the experimental diets, while postprandial samples taken after 58 – 67 days, and ^{1}H NMR spectra were acquired on these. Principal component analysis on the obtained NMR spectra demonstrated clear effects of diet on the plasma metabolite profile, and partial least squares regression discriminant analysis on the spectra revealed that the intensity of the spectral region at 3.29 ppm dominated the differentiation between the two diets, as the rye diet was associated with higher spectral intensity in this region. The 3.29 ppm signal is ascribed to $N(CH_3)_3$ protons in betaine, which may be an important contributor to the health promoting effects of rye.

Keywords: Betaine / Cereals / ^{1}H nuclear magnetic resonance / Metabolomics / Whole-grain rye

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1 Introduction

Evidence has been obtained that wholegrain cereals are protective against a range of degenerative life style related diseases such as cardiovascular disease (CVD), certain types of cancers and type II diabetes [1 – 7]. Regarding the prevention of CVD, focus has been devoted to cereals with high contents of β-glucans (oats and barley), for which hypocholesterolemic effects have been demonstrated [8, 9]. Nevertheless, positive effects of rye breads, which are rich in dietary fibers [10], have also been demonstrated [11, 12]. The food factor responsible for the preventive effects of wholegrain and fiber-rich cereal fractions and the underlying mechanisms associated with the grain-induced cholesterol reduction are still far from fully understood. The ability of the fiber complex to increase gut viscosity and thereby delay and/or reduce absorption and reabsorption of nutrients and bile acids, as well as specific effects of fermentation products and bioactive components on regulation of cholesterol metabolism in the liver have previously been suggested as discussed by Lærke *et al*. [11].

Proton (^{1}H) NMR spectroscopy provides concurrent detection of all mobile hydrogen-containing molecules in a sample, thereby making it a strong, explorative technique. This has been clearly demonstrated in pharmaceutical studies, where it is widely recognized that NMR-based metabonomics on biofluids is an excellent tool for detection of biochemical effects without any *a priori* knowledge [13]. Within the field of nutrition, NMR-based metabonomics is slowly emerging as a tool for elucidating how diet elicits

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Abbreviations: CPMG, carr purcell meiboom gill; **CVD,** cardiolar vascular disease; **HDL,** high-density lipoproteins; **LDL,** low-density lipoproteins; **PCA,** principal component analysis; **PLS,** partial least squares; **PLS-DA,** partial least square regression discriminant analysis; **TSP,** 3-(trimethylsilyl-[2,2,3,3-$^2H_4$-])-1-propionate

Table 1. Ingredients of experimental diets (g/kg).

	Wheat flour diet	Wash out diet	Experimental bun diets[a)]	
			Wheat	Rye
Wheat flour	735	815	528	0
Rye whole meal	0	0	0	310
Rye bran	0	0	0	400
Cellulose	0	60	157	00
Whey protein concentrate	10	69	25	0
Yeast	0	0	20	20
Sugar	0	0	15	15
Egg powder	150	0	150	150
Rape seed oil	20	28	20	20
Lard	50	0	50	50
Cholesterol	5	0	5	5
Vit/Min mix[b)]	30	28	30	30

a) Before water addition and baking.
b) Providing in mg/kg diet: 6642 $Ca(H_2PO_4)_2$, 4122 NaCl, 16 580 $CaCO_3$, 286 $FeSO_4 \cdot 7H_2O$, 114 ZnO, 41 Mn_3O_4, 92 $CuSO_4 \cdot 5H_2O$, 0.3 KI, 0.8 $Na_2SeO_3 \cdot 5H_2O$, 2.1 retinoacetate, 0.03 cholecalciferol, 69 α-tocopherol, 2.52 menadione, 4.58 riboflavin, 12.59 D-pantothenic acid, 0.025 cyanocobalamine (B_{12}), 2.52 thiamin (B_1), 25.2 niacin, 3.78 pyridoxine (B_6), 0.063 biotin.

changes in the metabolite profile. Applications of NMR-based metabonomics on blood plasma and urine samples for elucidating the biochemical effects of dietary bioactive compounds such as epicatechin [14], isoflavones [15], chamomile [16] and tea phenols [17] have been reported. In addition, it has also been demonstrated that NMR-based metabonomics is valuable for elucidating how wholegrain and cereal fractions alter the urine and plasma metabolite profile of pigs [18] and rats [19]. In the study on pigs, NMR-based metabonomics unraveled that a wholegrain-rye based diet resulted in an increase in betaine content in the plasma, which was not foreseen, and which therefore demonstrated the strength of applying NMR as an explorative, non-targeted tool [18]. However, the experimental pigs and rats included in these studies were healthy animals, and it could be relevant to explore the response to wholegrain cereals also in animals representing a hypercholesterolemic condition. Therefore, in the present study the effects of high-fiber rye bread intake on the plasma metabolite profile of hypercholerosterolemic pigs were investigated and compared with wheat bread intake at similar dietary fiber level in an intervention study using proton NMR-based metabonomics.

2 Materials and methods

2.1 Diets

The study included two experimental diets: (i) a high-fiber rye bread-based diet and (ii) a high-fiber wheat bread-based diet, both enriched with fat and cholesterol. Prior to administration of the high-fiber diets (high-fiber buns), the pigs were fed a low-fiber high-fat wheat flour-based diet (Table 1). In order to select for pigs responding to a high fat and cholesterol intake, the low-fiber diet was added 0.5% cholesterol plus 15% egg powder resulting in a total cholesterol content of ~1.25% during the first 2 wk. This was followed by 3 wk on a wheat flour-based low-fat wash-out diet containing 6% vitacel WF 600 (LCH A/S, Denmark). The experimental diets consisted of high-fiber buns either made of rye or wheat flour. The rye buns contained whole-kernel flour and rye bran, while the wheat buns contained wheat flour with added purified wheat fiber high in cellulose (Vitacel WF 600) in order to obtain same dietary fiber level [11] (Table 2). The wheat buns were produced at Cerealia Unibake, Denmark. The rye buns were made at Holstebro Technical College, Denmark. Bread buns were stored at –20°C until consumption.

Table 2. Content of macronutrients and total plant lignans (per 100 g DM) in the experimental buns

	Wheat	Rye
Dry matter, g per g fresh weight	66.1	68.0
Energy, MJ	21.10	21.16
Ash, g	3.9	5.0
Protein, g	18.2	17.2
Fat, g	15.3	15.9
Starch and sugar, g	43.7	38.4
DF[a)], g	19.4	20.3
NSP, g	17.3	16.3
Klason lignin, g	2.2	4.0
Lignans, μg	124.0	4152.7

a) DF, dietary fiber calculated as the sum of NSP and Klason lignin.

2.2 Experimental design and animals

Pigs from the swine herd at the Faculty of Agricultural Sciences, Foulum, Denmark, were used for the study. At a body weight of about 70 kg, 30 female pigs were fed the low-fiber, high-fat wheat diet containing cholesterol at a level 2 × 1 kg *per* day. After 2 wk on this diet, a fasting blood sample was taken from *vena jugularis* of each pig by veno-puncture, and a total of 17 hyper-responders (>3.5 mmol/L) were chosen as study subjects as described in detail by Lærke *et al*. [11]. For a sub-group of 12 pigs, the design was as follows: the following 3 wk, the pigs were fed the wash-out diet at a level of 2 kg/d for 2 wk increasing to 2.5 kg/d in the third week. In the fourth week, the pigs were randomly divided into two groups of six animals each and introduced to the two experimental diets consisting of the high-fiber rye or high-fiber wheat, respectively. During the 10 wk experimental period, the pigs were initially fed 1 kg diet *per* meal increasing to 1.5 kg per meal during the

last 3.5 wk. Fasting blood samples of the jugular vein were taken by veno-puncture 2 days before dietary intervention (termed d 0), on day 8 and 12 after transfer to the experimental diets. Sampling from two pigs is missing on day 12. After 65 – 67 day on the experimental diets the pigs, which now had an average body weight of 138.3 ± 2.4 kg, were anesthesized and 3 h post prandial blood samples were taken followed by euthanasia with an overdose of pentobarbital as described by Lærke *et al*. [11]. For the remainder five pigs, plasma samples were only collected at this last sampling point, *i.e*., no fasting blood samples were obtained. Otherwise, the experimental procedure deviated only from the procedure described above by a lower fiber level in the 1 wk shorter wash-out period, and by a 1 wk shorter experimental period [11]. Plasma samples for clinical chemical analyses were stored frozen at −20°C until analyses, while samples for NMR were stored at −80°C.

The animal experiments complied with the guidelines of the Danish Ministry and Justice with respect to animal experimentation and care of animals under study.

2.3 Clinical chemical analyses

Plasma glucose, triglycerides, total cholesterol, low-density lipoproteins (LDL) and high-density lipoproteins (HDL) cholesterol were analyzed using an auto analyzer, ADVIA 1650® Chemistry System (Bayer Corporation, Tarrytown, NY, USA) using human standards and calibration materials. Plasma glucose was determined according to the glucose hexokinase II method [20]. Triglycerides were analyzed by determination of glycerol after hydrolyses of mono-, di- and triglycerides according to Fossati and Prencipe [21]. Total cholesterol was determined by the enzymatic hydrolyses of cholesterol esters, oxidation of free cholesterol and concomitant liberation of hydrogen peroxide [22], and LDL and HDL cholesterol after selective isolation and liberation according to the principles of Okada *et al*. [23] and Izawa *et al*. [24], respectively. Plasma enterolactone (Enl) was analyzed by time-resolved fluoroimmunoAssay (TR-FIA) (Labmaster Diagnostics, Turku, Finland) using a 1,2,3,4 DELFIA research Fluorometer (Wallac, Finland). A correction factor of 0.80 was used to account for losses during hydrolysis and extraction [25, 26].

2.4 NMR measurements

The NMR measurements were performed at 300 K on a Bruker Avance 400 NMR Spectrometer (Bruker BioSpin), operating at a ^{1}H frequency of 400.13 MHz, and equipped with a standard 5 mm HX inverse probe. Prior to the measurements, plasma samples ($n = 51$) were thawed and 200 µL aliquots were mixed with 500 µL D_2O. Sodium trimethylsilyl-[2,2,3,3-$^{2}H_4$]-1-propionate (TSP) was added as an internal chemical shift reference (0.36 mg/mL). ^{1}H NMR spectra of plasma samples were obtained using a Carr-Purcell-Meiboom-Gill (CPMG) delay added in order to attenuate broad signals from high Mr components. The total CPMG delay was 40 ms and the spin-echo delay was 200 µs. Water suppression was achieved by irradiating the water peak during the relaxation delay of 2 s. A total of 128 transients of 8 K data points spanning a spectral width of 24.03 ppm were collected. An exponential line-broadening function of 3 Hz was applied to the free induction decay (FID) prior to Fourier transform (FT). All spectra were referenced to the TSP signal at 0.0 ppm.

2.5 Analysis of NMR data

The mean-normalized ^{1}H NMR spectra in the region 10.0 – 5.2 ppm and the region 4.6 – 0.5 ppm were used for further data analysis. The spectra were segmented into regions of 0.03 ppm width and the integral of each region was calculated. The reduced spectra consisting of 300 integrated regions were normalized to the whole spectrum to remove any concentration effects. Multivariate data analysis was performed using the Unscrambler software version 9.6 (Camo, Oslo, Norway). Principal component analysis (PCA) was applied to the centered data to explore any clustering behavior of the samples, and partial least square regression discriminant analysis (PLS-DA) was performed to explore intrinsic biochemical dissimilarities between predefined sample classes (Rye diet *vs.* wheat diet). During all regressions, Martens uncertainty test [27] was used to remove noisy variables, and all models were validated using full cross-validation.

3 Results

Typical proton NMR spectra obtained on the plasma samples are shown in Fig. 1. Several signals have been tentatively assigned, and some of these assignments are presented in the Figure. PCA, which is an unsupervised method, was performed on the pre-processed ^{1}H NMR spectra obtained on day 0, 8, 12 and at slaughter, and the resulting plots of score 1 *versus* score 2 for mean-centered data are shown in Fig. 1a – d with each spectrum represented by a single data point. Before the intervention, samples are randomly distributed in the score plot (Fig. 2a), while at all later sampling times, a clear separation of samples according to diet is observed, and the separation seems to be more marked with time (Fig. 2b – d). PLS-DA, which is focused on discriminating variation between pre-defined classes, was performed on the ^{1}H NMR spectra to investigate the metabolic differences in plasma profile between the two diets at the different sampling times during the intervention. The PLS-DA scores plots show a clear separation of rye and wheat plasma along the first component at day 8, day 12 and at slaughter (Fig. 3b – d), and the first X-loading is therefore examined to explore the regions in the NMR spec-

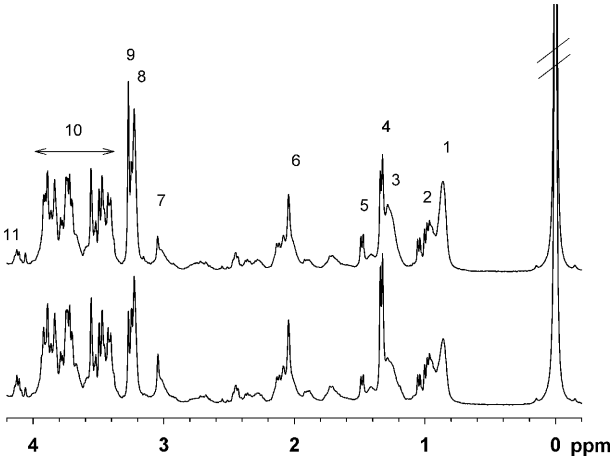

Figure 1. Representative proton NMR spectra obtained on plasma samples taken on day 12 from a pig on rye diet (top) and a pig on wheat diet (bottom), respectively. The main resonances observed are assigned as follows: 1 CH_3 protons in fatty acid chains; 2 CH_3 protons in leucine, isoleucine, and valine; 3 CH_2 protons in fatty acid chains; 4 CH_3 protons in lactate; 5 CH_3 protons in alanine; 6 CH_3 protons in *N*-acetyl groups; 7 CH_3 protons in creatine; 8 $N(CH_3)_3$ protons in choline; 9 $N(CH_3)_3$ protons in betaine; 10 protons from various sugars and amino acids; 11: CH protons in lactate. Signal at 0 ppm is the internal TSP standard.

trum that differ between the two diets (Fig. 2f–h). At all time points, a signal at 3.29 ppm, which is higher in intensity in rye samples, is dominating the differentiation between wheat and rye samples. The 3.29 ppm signal is tentatively assigned to methyl protons arising from $N(CH_3)_3$ in betaine. After 8 days diet intervention, higher intensities of signals at 1.27 and 5.28 ppm in rye samples, which are tentatively assigned to CH_2 and CH=CH protons in triglycerides, respectively, are also contributing to a minor degree to the discrimination between rye and wheat samples. In addition, lower intensities of signals in the region ~3.4–4.0 ppm in rye samples, which are tentatively assigned to CH protons in sugars, are also contributing at a lower level to the discrimination between rye and wheat samples after 8 days of diet intervention (Fig. 3f). After 12 days of intervention, a higher intensity of the 1.27 ppm signal in rye samples, which is ascribed to CH_2 protons in triglycerides, is also contributing to the discrimination at a lower level (Fig. 3g). At slaughter time, besides the dominating contribution from betaine (3.29 ppm), lower intensities of signals at 0.98 and 2.06 ppm in rye samples also contribute to a minor degree to the discrimination. The 0.98 ppm signal is tentatively assigned to CH_3 protons in triglycerides, while the 2.06 ppm signal is assigned to CH_2–CH=CH in triglycerides.

In order to investigate the relationship between the NMR spectra and biochemical data (cholesterol, HDL, LDL, triglycerides, glucose, Enl), partial least squares (PLS) regressions were carried out with the ^{1}H NMR CPMG spectra independent variables as predictors in regression models with the various metabolite concentrations determined biochemically as *y*-variables. The results of these regression models are summarized in Table 3, and Fig. 4 shows the X-loadings from the regressions. The loadings demonstrate that signals assigned to CH_3– (~0.9 ppm) and CH_2–groups (~1.3 ppm) in fatty acid chains are positively correlated to

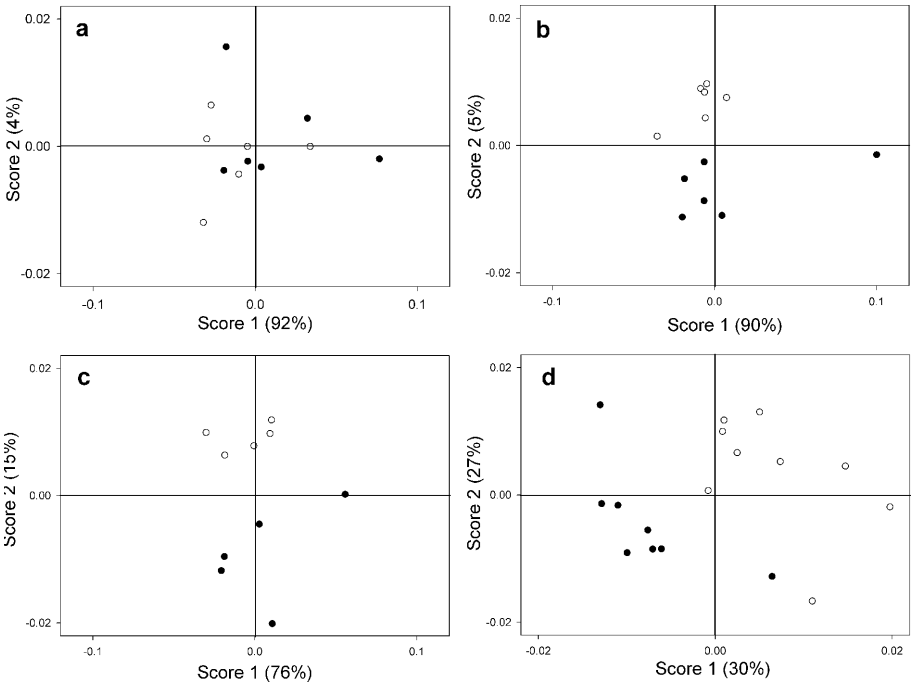

Figure 2. PCA score plots obtained on NMR spectra acquired on samples collected at: a) day 0, b) day 8, c) day 12, and d) at slaughter. Closed circles represent wheat diet, and open circles represent rye diet.

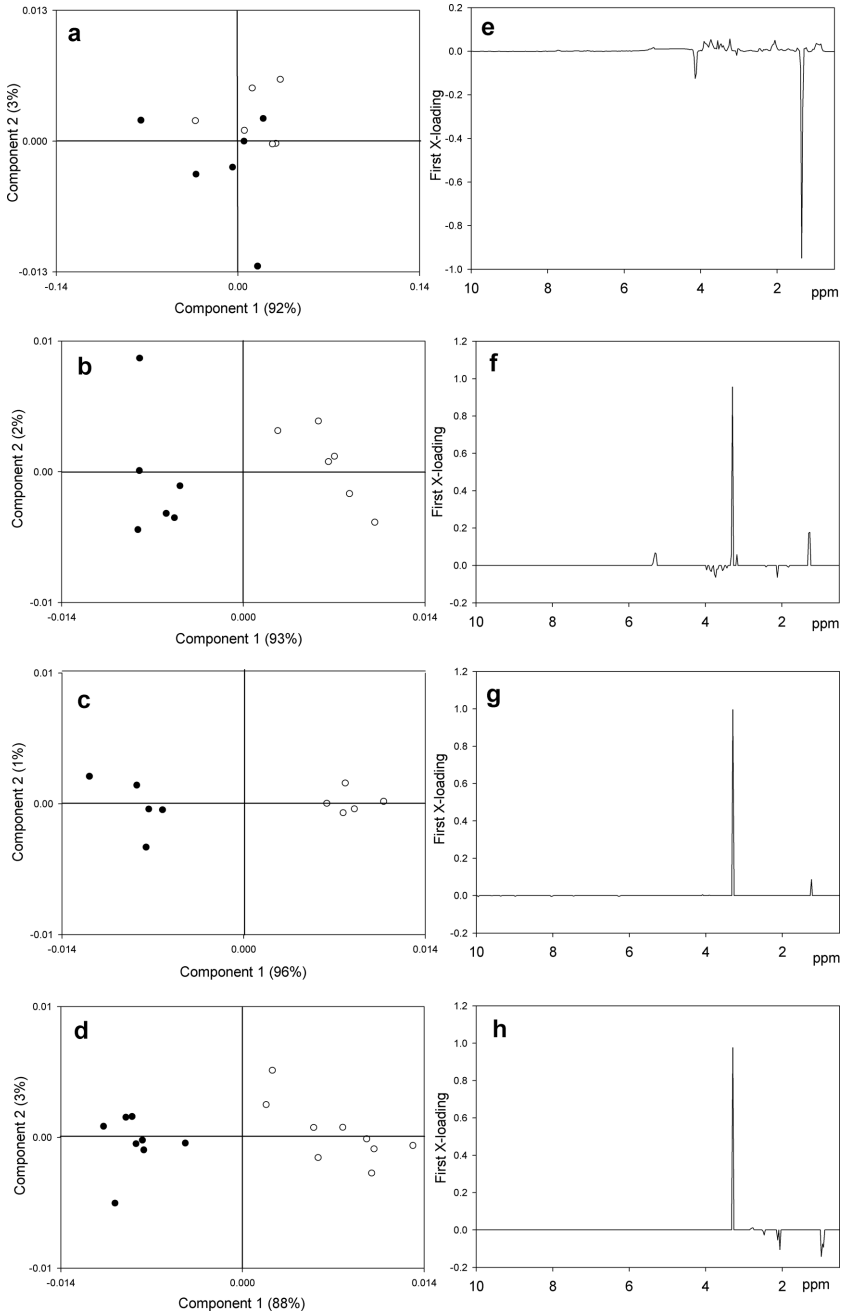

Figure 3. PLS-DA scores plot from analysis of NMR spectra acquired on wheat and rye plasma samples collected at: a) day 0, (b) day 8, (c) day 12, and (d) at slaughter; and the corresponding first X-loadings (e – h). Closed circles represent wheat diet, and open circles represent rye diet, and positive values in the X-loadings indicate higher intensity in rye samples compared with wheat samples.

cholesterol ($p = 0.10$), triglyceride ($p = 0.17$), and LDL ($p = 0.10$) concentrations and more weakly to HDL ($p = 0.35$) concentrations (Fig. 4). The characteristic 3.3 ppm signal arising from betaine is positively correlated to Enl ($p = 0.001$). In the ~3 – 4 ppm region, which contains signals from various CH-groups in sugars [28], positive correlations to glucose are demonstrated.

4 Discussion

A relationship between the consumption of wholegrain cereals and incidence of life-style related diseases, *i.e.* CVD, certain types of cancers and diabetes, has been established [1 – 7]. However, the underlying mechanisms responsible for this relationship are still not fully understood. This study is the first to report the use of ^{1}H NMR-based metabonomics for exploring the effect of high-fiber rye bread in hypercholesterolemic pigs. Human and pigs have similar apolipoprotein profiles when pigs are fed a high fat diet [29, 30], and under experimental conditions pigs develop complex atherosclerotic lesions morphologically similar to those found in humans in just 3 – 8 months [31]. The pig is therefore considered to be a suitable model for humans for studying the hypocholesterolemic effects of

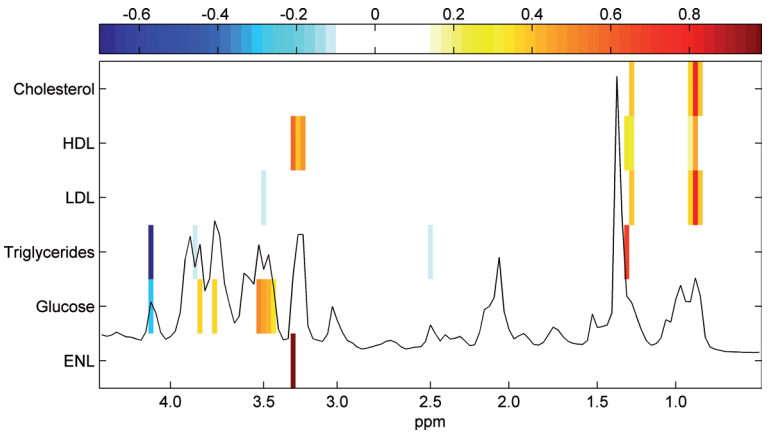

Figure 4. Loadings from PLS regressions with ^{1}H CPMG NMR spectra obtained on plasma as *x*-variables and various biochemical analyses as *y*-variables. ENL: enterolactone. Color scale bar in top shows value of the loadings. The loadings express how much the various *x*-variables contribute to the prediction of the *y*-variable. *X*-variables that are important in the prediction of *y* have high loadings (*i.e.*, close to 1 or −1).

Table 3. Performances of PLS regression models with plasma ^{1}H CPMG NMR spectra as X-variables and plasma metabolite concentrations as *y*-variable ($n = 51$)

Metabolite	Reference value interval	RMSECV[a)]	PLS[b)]	R[c)]
Cholesterol (mmol/L)	2.0–15.8	2.1424	2	0.78
HDL (mmol/L)	0.65–2.59	0.1280	5	0.95
LDL (mmol/L)	0.89–9.62	1.5186	3	0.72
Triglycerides (mmol/L)	0.11–2.79	0.3347	2	0.67
Glucose (mmol/L)	3.86–7.81	0.2781	6	0.89
Enterolactone (μmol/L)	6.7–989.1	204.01	1	0.74

a) Root mean square error of cross validation.
b) Number of PLS components used.
c) Correlation coefficient.

dietary fiber and wholegrain cereals [11, 30]. PCA on ^{1}H NMR spectra acquired on plasma samples revealed a clear separation of fiber-enriched wholegrain rye and non-wholegrain wheat samples both on day 8, day 12, and at slaughter, implying a significant effect on the plasma metabolite profile of the two diets (Fig. 2). In order to investigate the differences in the plasma metabolite profile of the fiber-enriched wholegrain rye and non-wholegrain wheat samples, PLS-DA, which is a supervised method, was performed on spectra obtained on rye and wheat samples (Fig. 3). Both on day 8, day 12 and at slaughter, the X-loadings revealed that PLS-DA could clearly discriminate between wholegrain rye and non-wholegrain wheat samples mainly because of pronounced increases in spectral intensities of a signal at 3.29 ppm in rye plasma samples compared with wheat samples. 3.29 ppm corresponds to the chemical shift value reported for different $N(CH_3)_3$ groups [28], and in an investigation on pigs also fed a high-fiber rye diet we have previously shown that this response should be ascribed to betaine using LC-MS and spiking experiments [18]. Accordingly, the present study demonstrates that for both normal and hypercholesterolemic pigs, the same effects of high-fiber rye intake are dominating the plasma metabolite profile obtained by ^{1}H NMR spectroscopy. In addition, the present study manifests that betaine seems to be a biomarker for intake of fiber-rich tissues present in a wholegrain rye-based diet, as it was dominant at all sampling times during the intervention. However, as other food sources such as beets, broccoli, spinach, and shellfish are also sources of betaine, it remains unknown if betaine can be considered a general biomarker for rye intake in a mixed diet, where alkylresorcinols may be a better biomarker [32, 33]. Previous studies have shown that dietary betaine is absorbed and causes increases in serum concentrations [34–37], and it is likely that the present findings reflect a direct betaine absorption. It is worth noticing that betaine serves a methyl donor in the betaine-homocysteine methyltransferase reaction that converts homocysteine to methionine [38], and an inverse relationship between betaine supplementation and plasma levels of homocysteine has been demonstrated [39, 40]. As plasma homocysteine is a risk factor for CVDs [41], betaine can ultimately also be expected to be involved in the prevention of CVD through this pathway. This is also consistent with the findings of a recent epidemiological study, which revealed that plasma betaine is inversely associated with serum non-HDL cholesterol, triglycerides, BMI, percent body fat, waist circumference, systolic, and diastolic blood pressure [42]. Accordingly, in view of the physiological significance of betaine, the present findings demonstrating a strong relationship between the intake of rye and plasma betaine must be considered particularly important.

In addition to the dominating effect of the rye buns on plasma betaine, PLS-DA regressions also revealed contributions from fatty acyl protons at 0.9 and 1.3 ppm to the discrimination of the two diets (Fig. 3). However, which exact effects that these fatty acid chain contributions represent remains unclear. A large proportion of fatty acyl protons giving rise to signals at the 0.9 and 1.3 ppm positions is bound in lipoproteins, and the contributions could possibly reflect a diet-induced alteration in the composition of lipoproteins and/or the HDL/LDL-fraction. This would be consistent with a previous metabolic characterization of liver

biopsies from the animals included in the present study, which pointed toward an effect of the diet on cholesterol metabolism in the liver [43]. Nevertheless, the PLS regressions between the NMR metabolite profiles and plasma lipid parameters reveal that intensities of the 0.9 and/or 1.3 ppm lipid signals correlate with more chemical data: HDL, LDL, total cholesterol, and total triglyceride content (Fig. 4). This implies that it is difficult to further elucidate if the effect of betaine on the lipid signals specifically involves cholesterol or lipid metabolism. At slaughter the effects of rye buns on NMR signals assigned to lipids were distinct from the other sampling times, which probably should be ascribed to the fact that samples at slaughter were not fasting samples.

PLS regressions between ^{1}H NMR spectra acquired on plasma and biochemical determinations of various plasma metabolites established correlations between NMR signals from the CH_2- and CH_3- groups in fatty acyl chains and biochemical determinations of HDL, LDL, total cholesterol, and total triglyceride content (Fig. 4). In addition, correlations between NMR signals from CH protons in glucose and glucose concentrations determined biochemically were demonstrated. Intriguingly, the PLS regressions also revealed correlations between the NMR plasma metabolome and Enl, which is a compound present in a concentration that is not directly measurable in plasma with the applied ^{1}H NMR method. Enl was found to be highly correlated to the intensity of the betaine signal, which probably should be ascribed to the fact that intake of wholegrain rye increases both plasma content of betaine and Enl.

In conclusion, using NMR-based metabonomics as an explorative approach and hypercholesterolemic pigs representing a human model, the present studies disclosed some metabolic effects of a high-fiber rye diet, especially on the content of betaine in plasma. The observed effect on plasma betaine content may contribute to the health benefits of a high dietary intake of wholegrain, and further studies emphasizing the potential beneficial role of betaine in the health-promoting effects of wholegrain cereals are needed. Moreover, for understanding all mechanisms involved in the health-promoting effects of high-fiber diets, supplementary studies including LC-MS-based metabonomics could also be of great interest to investigate if it might disclose additional metabolic effects in relation with low-concentration metabolites and/or new biomarkers of wholegrain rye consumption that are not detected by ^{1}H NMR spectroscopy.

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The authors have declared no conflict of interest.

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