Research report

Second meal effect on appetite and fermentation of wholegrain rye foods

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Background: Wholegrain rye has been associated with decreased hunger sensations. This may be partly mediated by colonic fermentation. Sustained consumption of fermentable components is known to change the gut microflora and may increase numbers of saccharolytic bacteria. Objective: To investigate the effect of wholegrain rye consumption on appetite and colonic fermentation after a subsequent meal. Methods: In a randomized, controlled, three-arm cross-over study, twelve healthy male subjects consumed three iso-caloric evening test meals. The test meals were based on white wheat bread (WBB), wholegrain rye kernel bread (RKB), or boiled rye kernels (RK). Breath hydrogen excretion and subjective appetite sensation were measured before and at 30 min intervals for 3 h after a standardized breakfast in the subsequent morning. After the 3 h, an ad libitum lunch meal was served to assess energy intake. In an in vitro study, RKB and RK were subjected to digestion and 24 h-fermentation in order to study SCFA production and growth of selected saccharolytic bacteria. Results: The test meals did not differ in their effect on parameters of subjective appetite sensation the following day. Ad libitum energy intake at lunch was, however, reduced by 11% (P < 0.01) after RKB and 7% (P < 0.05) after RK compared with after WBB evening meal. Breath hydrogen excretion was significantly increased following RKB and RK evening meals compared with WWB (P < 0.01 and P < 0.05, respectively). Overall, RKB and RK were readily fermented in vitro and exhibited similar fermentation profiles, although total SCFA production was higher for RK compared with RKB (P < 0.001). In vitro fermentation of RKB and RK both increased the relative quantities of Bifidobacterium and decreased Bacteroides compared with inoculum (P < 0.001). The C. coccoides group was reduced after RKB (P < 0.001). Conclusion: Consumption of wholegrain rye products reduced subsequent ad libitum energy intake in young healthy men, possibly mediated by mechanisms related to colonic fermentation.

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Introduction

Obesity and associated co-morbidities constitute an increasing problem worldwide ([World Health Organization, 2013]). Observational studies suggest a role of wholegrain in combating obesity, as wholegrain consumption has been associated with lower body weight gain ([Koh-Banerjee et al., 2004; Liu et al., 2003]), although this was not confirmed in a meta-analysis on the effect of wholegrain on body weight based on intervention studies. However, a small beneficial effect on body fat percentage was found ([Pol et al., 2013]). It is proposed that increasing wholegrain intake may enhance satiety sensation and thus may lead to a reduced energy intake and a lower body weight in the long term ([Giacco, Della, Luongo, & Riccardi, 2011]).

In Scandinavian countries, rye is among the most commonly consumed cereals ([Frølich, Åman, & Tetens, 2013]). Wholegrain rye is a rich source of fermentable dietary fibers, such as arabinoxylans, β-glucan, fructans, and resistant starch ([Frølich et al., 2013; Isaksson et al., 2011]), where substantial amounts of resistant starch are found in more intact food structures, such as whole and cracked kernels ([Liljeberg, 2002; Nilsson, Östman, Granfeldt, & Björck, 2008a]). In the colon, saccharolytic bacteria, such as Bifidobacterium and Bacteroides ([Duncan, Louis, Thomson, & Flint, 2009]), utilize these ferment-
able components with a resulting production of short-chain fatty acids (SCFA) and gasses, such as hydrogen, carbon-dioxide, and methane (Wong, de Souza, Kendall, Eamam, & Jenkins, 2006). Regular wholegrain consumption likely leads to an increase in the abundance of saccharolytic bacteria as a high availability of fermentable substrates will promote their growth (Zhou et al., 2008).

Fermentation may be one of the mechanisms by which wholegrain consumption influences appetite, as SCFA have been reported to enhance the production of satiety-inducing hormones such as GLP-1 and PYY (Tolhurst et al., 2012; Zhou et al., 2008). However, in order for fermentation to occur, the fermentable component needs to reach the colon, hence fermentation of most cereals takes place >4 h after meal ingestion (Nilsson et al., 2008a). Therefore, effects of wholegrain fermentation on appetite are not expected to occur until the subsequent meal (Isaksson et al., 2011; Isaksson, Sundberg, Aman, Fredriksson, & Olsson, 2008).

It is difficult to assess the extent of fermentation as well as amount and type of produced SCFA. Human intervention studies often use breath hydrogen excretion as a measure of fermentation (Johansson, Nilsson, Östman, & Björck, 2013; Nilsson, Östman, Holst, & Björck, 2008b; Rosen, Östman, & Björck, 2011). However, as this does not provide information on the proportion of the different types of SCFA produced and bacteria stimulated, in vitro fermentation with human inoculum constitutes a method to investigate colonic fermentation in more detail (Topping & Clifton, 2001), although only comparable to a limited extent to in vivo situations.

In the present pilot study we investigated whether a wholegrain rye kernel bread and boiled rye kernels consumed late in the evening influenced subjective appetite sensation and breath hydrogen excretion after a standardized breakfast meal at the following morning as well as ad libitum energy intake at a subsequent lunch meal in healthy young subjects. The methodology of the postprandial part of the study was similar to that applied in the human intervention studies of the research center “Gut, Grain and Greens” (3G) (Ibrügger, & Björck, 2008b). Therefore, effects of wholegrain fermentation on appetite are not expected to occur until the subsequent meal (Isaksson et al., 2011; Isaksson, Sundberg, Aman, Fredriksson, & Olsson, 2008).

Methods

Subjects

Twelve healthy 18–65 year-old men with normal BMI (18–25 kg/m²) were recruited via advertising at university campuses at the University of Copenhagen. Exclusion criteria were known chronic diseases, regular intake of pre- or probiotics, use of dietary supplements up to one month before study start, smoking, high level of physical activity (>10 h/wk) and participation in other studies. All participants gave written informed consent to participate in the study. The study did not require ethic approval, as no biological samples were taken. Data collection was in accordance with the Data Protection agency (2007-54-0269) and according to the Helsinki declaration. The study was carried out at the Department of Nutrition, Exercise and Sports, Faculty of Science, University of Copenhagen, Denmark.

Experimental design and procedure

The human intervention study was designed as a randomized, controlled, cross-over study. The effect of three different, isocaloric grain-based evening meals, including wholegrain rye kernel bread (RKB), boiled rye kernels (RK), or white wheat bread (WWB) as a reference, on appetite sensation and breath hydrogen excretion during the next morning was investigated. Participants were allocated to one of three different orders of test meals in a Williams design, taking first-order carry-over effects into account. This was done by simple randomization using a web-based program (http://www.randomization.com). Each meal test was completed over a two-day period and separated by at least a three-day wash-out period.

Day 1: Evening test meal

On day 1, participants were instructed not to consume any food after 17:00 and to drink 500 ml of water until arrival at the department at 21:15. During the course of the whole day, participants had to consume a diet low in dietary fibers and in order to check compliance they were asked to note down all consumed food items in a food diary. Furthermore, they were not allowed to drink alcohol and perform vigorous physical activity during the day. After arrival at the department, breath hydrogen excretion and subjective appetite sensation were measured, using visual analogue scales (VAS). The test evening meals were served at 21:30 together with 300 ml water and subsequently appetite sensation was assessed again. Participants were instructed to drink 500 ml of water till the next morning.

Day 2: Standardized breakfast and ad libitum lunch

In the morning of day 2, participants arrived in a calm mode at the department at 7:30, after an overnight fast (10 h). Upon arrival, fasting breath hydrogen and appetite sensation were measured. At the first examination, participants had their height measured to the nearest 0.5 cm by a wall-mounted stadiometer (Seca) and were weighed to the nearest 0.05 kg (Lindell Tronic 8000) in light clothing and with an empty bladder. At 8:00 a standardized breakfast meal was served consisting of white wheat bread, butter, cheese, jam, a pastr y and 200 ml water (approximately 3000 kJ, 52% fat, 40% carbohydrates, 8% protein). Breath hydrogen excretion and appetite sensation were measured every 30 min over the following 180 min. After 90 min, 200 ml of water were served. At 11:00, an ad libitum lunch meal was provided in order to assess voluntary energy intake. After the meal, appetite sensation was registered again.

Composition of evening test meals

The amount of test product of the test meals was based on 50 g carbohydrate according to package labeling. Meals were isocaloric (2.5 MJ) and contained either RKB, RK or WWB together with milk (per 100 g: 190 kJ, 4.7 g carbohydrate, 1.5 g fat, 3.4 g protein), cheese (per 100 g: 1343 kJ, 0.1 g carbohydrates, 25.0 g fat, 23.5 g protein), and water (Table 1). RK were boiled for 35 min at day 1 and

<table>
<thead>
<tr>
<th>Component</th>
<th>WWB meal</th>
<th>RKB meal</th>
<th>RK meal</th>
</tr>
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<tbody>
<tr>
<td>Grain part (unprepared) (g)</td>
<td>–</td>
<td>–</td>
<td>83</td>
</tr>
<tr>
<td>Grain part (as eaten) (g)</td>
<td>111</td>
<td>143</td>
<td>147</td>
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<tr>
<td>Milk (ml)</td>
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<td>250</td>
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<tr>
<td>Cheese (g)</td>
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<td>64</td>
<td>65</td>
</tr>
<tr>
<td>Water (ml)</td>
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<td>300</td>
<td>300</td>
</tr>
<tr>
<td>Energy (kJ)</td>
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<td>2475</td>
<td>2475</td>
</tr>
<tr>
<td>Carbohydrates (g)</td>
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<td>62</td>
<td>62</td>
</tr>
<tr>
<td>Fat (g)</td>
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<td>21</td>
<td>22</td>
</tr>
<tr>
<td>Protein (g)</td>
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<td>32</td>
<td>31</td>
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<tr>
<td>Dietary fiber (g)</td>
<td>3</td>
<td>11</td>
<td>12</td>
</tr>
<tr>
<td>Resistant starch (g)</td>
<td>4.0</td>
<td>7.6</td>
<td>11.1</td>
</tr>
</tbody>
</table>

RK, boiled rye kernels; RKB, wholegrain rye kernel bread; WWB, white wheat bread.

a According to product labeling.
b Analyzed in vitro.
stored at 5 °C until use. Prior to consumption they were re-heated covered with kitchen film in the microwave for 2 min. RKB was served cold.

Resistant starch analysis

Resistant starch was analyzed in samples of the RKB, RK and WWB products using a downscaled modified procedure according to Åkerberg, Liljeberg, Granfeldt, Drews, and Björck (1998), including an in vivo churning step followed by an in vitro digestion procedure. One healthy individual was used to chew all the test samples in replicates. The chewed samples were immediately frozen in liquid nitrogen and stored at −20 °C. Upon analysis the samples were thawed at 37 °C and in vitro digestion was performed as described in Åkerberg et al. (1998). Hereafter, precipitation of non-digested material was performed by addition of preheated (60 °C) ethanol. After 1 h the samples were centrifuged (10 min, 4500 G) and the supernatant removed. To completely remove soluble sugar the pellets were washed three times at 37 °C with 75% ethanol with intermitting incubations for 15 min followed by centrifugation (10 min, 4500 G). Thereafter, the pellets were dried overnight (105 °C) and remaining starch was analyzed as described by Shaik et al. (2014) (Shaik et al., 2014).

Measurements

Habitual dietary fiber intake

During one of the examination days, participants filled out a food frequency questionnaire (FFQ) assessing their habitual dietary fiber intake. In the FFQ, participants stated portion size and frequency of their intake of dietary fiber-rich food items during the last month. The habitual daily dietary fiber intake of the participants was estimated by using the average dietary fiber content of a range of products within each food item category. The FFQ was validated in 125 individuals against a 7-day weighed food record (Pearson’s correlation 0.63; P < 0.001) (Vuholm et al., submitted).

Breath hydrogen excretion

Breath hydrogen was measured in exhaled breath as a measure of colonic fermentation using a handheld Gastro+ Gastrolyzer® (Bedfont Scientific Ltd.). Participants were instructed to breathe in deeply, hold their breath for 15 sec and then exhale at a steady pace into the cardboard mouthpiece of the device until their lungs felt empty.

Subjective appetite sensation

Subjective appetite sensation, including hunger, satiety, fullness, and expected prospective food intake, was measured using 100 mm VAS with the most positive and the most negative rating in a range of products within each food item category. The FFQ was validated in 125 individuals against a 7-day weighed food record (Pearson’s correlation 0.63; P < 0.001) (Vuholm et al., submitted).

Ad libitum energy intake

To assess ad libitum energy intake, a large homogenous pasta Bolognese lunch meal was served (approximately 8 MJ, 54 E% from carbohydrates, 30 E% from fat, 15 E% from protein). To ensure reproducibility, any deviations in volume of the meal during preparation were adjusted with water. At each meal test the participants were instructed to eat until comfortably full and were not allowed to read, talk, listen to music, or other kinds of distracting activities while eating the ad libitum meal. All foods not eaten, whether on the plate or in the boiling pan, were weighed back and the amount of food and energy consumed was calculated. With the ad libitum meal, 300 ml of water was served and participants were instructed to drink it all.

In vitro digestion of RKB and RK

RKB and RK product samples were in vitro digested in order to simulate physiological conditions in the subsequent fermentation. RKB (50 g) and freshly prepared RK (50 g unprepared) were mixed with 190 ml of MilliQ water and blended for approximately 30 sec by a mixer until a similar consistency as after mastication, as monitored by visual inspection. The mixture was transferred to a glass screw topped bottle, blended with 5.2 ml α-amylase (Sigma) (78 mg α-amylase in 1 M CaCl₂, 25 ml, pH 7) and incubated on an oscillatory shaker (250 rpm, 37 °C, 15 min). Following, 17 ml pepsin (Sigma) (10.8 g pepsin in 0.1 M HCl, 100 ml) was added, the pH was lowered to pH 2.0 with 6 M HCl and the mixture incubated with shaking (250 rpm, 37 °C, 90 min). The pH was adjusted to pH 7.0 with 6 M NaOH and the following solutions were added: 2.1 ml pancreatic (Sigma) (400 mg pancreatin in MilliQ water, 10 ml), 2.1 ml mineral solution (0.441 g CaCl₂,2H₂O, 0.122 g MgCl₂,6H₂O MilliQ water, 10 ml), 6.9 ml amyloglucosidase (Sigma) (2800 U in MilliQ water, 20 ml), 22 ml bile acid (Sigma) solution (22.5 g bile acids in 150 mM NaHCO₃), and 1.7 ml isopropanol. The volumes were adjusted to 150 ml and the reaction was incubated on an oscillatory shaker (250 rpm, 37 °C, 30 min). The samples were transferred to a molecular weight cut-off dialysis tubing system (Standard Grade Regenerated Cellulose membrane 1 kDa MWCO, Spectra/ Por) and dialyzed against 10 M NaCl under gentle stirring at 37 °C to remove digested products of low molecular mass. After overnight dialysis, the dialysis outer salt solution was changed twice and dialysis continued for 3 h, respectively. Dialyzed samples were lyophilized by freeze-drying and stored dry prior to in vitro fermentation.

In vitro fermentation

Fecal samples for the fermentation procedure were obtained from three healthy donors (28–35 y), with no history of gastrointestinal disorders and no treatment with antibiotics for at least four months before sample collection. Whole stools were collected in airtight containers and stored at 4 °C until delivery to the laboratory, where they were processed immediately. Prior to in vitro fermentation, fecal samples were homogenized in 50% glycerol (1:1 dilution) in an anaerobic cabinet (containing 10% H₂, 10% CO₂, and 80% N₂) and stored at −80 °C until use.

The freeze-dried in vitro digested RKB and RK products as well as inulin and cellulose as positive and negative control, respectively, were added to autoclaved minimal basal medium, prepared as described by Vigsnaes, Holck, Meyer, and Licht (2011), to give a final concentration of 10 g/l in a reaction volume of 50 ml. The reduced minimal medium with the added freeze-dried samples was inoculated with the thawed fecal slurry to a final concentration of 1% (w/v) feces. Fermentations of each carbohydrate source were run in triplicates with feces from each donor and the process was carried out in an anaerobic cabinet at 37 °C. Fermentation samples were rotated using a rocker (Multi Bio RS, BioSan) and not controlled for pH during the course of the fermentation. For the inoculum sample, 5 ml of the fecal slurry were mixed with basal medium. After 6 h and 24 h of fermentation, 5 ml of sample was taken and centrifuged at 13,000 rpm for 8 min. The supernatants were used for pH measurements and SCFA analysis and the pellets for extraction of bacterial DNA. Pellets and supernatants were stored at −21 °C until use.
Measurement of pH and SCFA analysis after in vitro fermentation

Measurement of pH was done in inoculum samples and in extracts after 6 h and 24 h of fermentation using a handheld pH meter (Thermo Scientific Orion 3 Star). Prior to quantification of SCFA, the collected supernatants were acidified to pH <2 with 17% H₃PO₄. The acidified samples were filtered through a 0.45 μm filter (Milipore) to remove bacterial cells. SCFA were analyzed using gas chromatography as previously described by Vigsnæs et al. (2011), with modification of the temperature gradient (105 °C for 3 min, then increased by 8 °C/min to 130 °C and thereafter at 45 °C/min to 230 °C which was held for 3 min) (Vigsnæs et al., 2011). The quantified SCFA were acetic, propionic, and butyric acid. The standards used in the SCFA analysis were the same acids as mentioned above with concentrations of 1–40 mM.

Characterization of bacterial composition after in vitro fermentation

Bacterial DNA was extracted from inoculum samples using the QiAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) with a bead-beater step in advance, as described by Leser, Lindecrona, Jensen, Jensen, and Moller (2000). The purified DNA was stored at –20 °C until use.

The purified bacterial DNA was amplified in optical grade 384-well plates by real-time qPCR using the ABI-Prism 7900 HT from Applied Biosystems. PCR reagents and DNA were loaded on the 384-well plates using an EpMotion pipetting Robot (Eppendorf). Primers specifically targeting 16S rRNA gene sequences of total bacteria, the phyla Bacteroidetes, Firmicutes, and Actinobacteria as well as of the genera Alistipes, Bacteroides, Lactobacillus, Bifidobacterium, Prevotella, and the C. coccoides group, respectively, were included in the qPCR analysis. The 16S rRNA primers used are listed in Supplemental Table S1 in the online version at doi:10.1016/j.appet.2014.05.026 of the online supplemental material. The amplification reactions were carried out as described by Bergström et al. (2012). The qPCR data were baseline corrected and N₀-values, representing initial concentrations of the specified 16S rRNA genes, were calculated using the LinRegPCR software (version 11.1, based on Ruijter et al., 2009). All results were calculated as means of duplicate N₀ estimations, equal values required. The relative quantities of gene targets encoding 16S rRNA sequences of the bacterial taxa were calculated using N₀ (bacterial target)/N₀ (total bacterial population).

Calculations and statistical analyses

All statistical analyses were performed by using R version 2.15.1 (The R Foundation for Statistical Computing, 2012). GraphPad Prism version 6.03 (GraphPad Software Inc., 2013) was used for preparing graphs and LatentTiX version 2.0 (LatentTiX™) for principal component analysis (PCA). All dependent variables were controlled for homogeneity of variance and normal distribution by investigation of residual plots and normal probability plots, respectively. Subjective appetite ratings and breath hydrogen excretion were analyzed using a repeated-measures ANCOVA with a Gaussian covariance structure in order to test whether there was an overall effect of treatment. In all analyses treatment order and the order × treatment-interaction term were included as fixed variables and participant as a random variable. The interaction term was removed when not significant in a maximum likelihood test. Evening breath hydrogen excretion and appetite ratings after the evening meal were included as covariates in analyses of breath hydrogen and appetite ratings, respectively. In order to test the hypothesis that fermentation affects appetite sensation by different mechanisms, Pearson correlations of breath hydrogen excretion at both fasting and 180 min with appetite parameters at fasting and 180 min as well as ad libitum energy intake were performed. Meal-induced differences in the ad libitum energy intake at lunch were tested using an ANOVA with order of treatment as fixed variable and participant as random variable. When effects of treatment were significant, post-hoc pairwise comparisons were made to test for differences between the treatments. As quantitative qPCR data and SCFA were not normally distributed, a non-parametric ANOVA with donor as random effect was performed for these data. Post-hoc pairwise comparisons were used to investigate differences among time points and products. Spearman correlations between bacterial species and pH were performed. The different bacterial taxonomic units and SCFA were subjected to PCA as a function of time (0, 6, and 24 h) and substate (RKB, RK, inulin, and cellulose) to generate an overview of the variation of the ability of the microbial communities to ferment the four carbohydrate sources at different time points. Data were visualized in two dimensions using a principal component Bi-plot. For analyses of the human intervention study level of significance was set to P < 0.05 and for the in vitro fermentation to P < 0.001. Data are presented as unadjusted mean ± SD unless otherwise stated.

Results

All twelve participants of the human study completed all meal tests. Participants were in average 25.6 ± 3.9 years of age with BMI of 23.1 ± 1.2 kg/m². According to the FFQ, their habitual dietary fiber intake was 26.2 ± 11.2 g/d, whereof 6.3 ± 3.7 g derived from fruits and vegetables, 17.7 ± 9.4 g from cereal products, and 2.3 ± 1.7 g from other products such as legumes, potatoes, and nuts. During the days of test evening meals participants adhered to a diet low in dietary fiber which was confirmed from review of food diaries.

Appetite

Subjective appetite sensation

Baseline VAS measurements of subjective appetites ratings in the evening before and after the evening test meal as well as in the morning before the standardized breakfast did not differ between treatments (data not shown). Repeated measures analyses did not show significant differences between any of the subjective appetite parameters after the standardized breakfast meal (P > 0.10). Due to the treatment-dependent patterns of appetite ratings observed visually, analyses of single time points were performed. These analyses showed a tendency towards a reduced hunger sensation in the late postprandial phase after RKB evening meal at 150 min (P = 0.05) and RK at 150 min and 180 min (P = 0.07 and P = 0.09, respectively) compared with WWB after the standardized breakfast. Similarly, participants tended to feel fuller at 120 min (P = 0.07) after RK evening meal compared with WWB and reported a lower rated prospective food intake at 180 min after RK compared with WWB (P = 0.04) (Fig. 1).

Ad libitum energy intake

There was an overall effect of treatment on voluntary ad libitum energy intake (P = 0.03). Ad libitum energy intake at lunch, 3 h after the standardized breakfast was reduced by 11% after RKB and by 7% after RK evening meals compared with WWB (P < 0.01 and P < 0.05, respectively) (Fig. 2).

Fermentation

Breath hydrogen excretion in human study

Repeated measures ANCOVA showed that breath hydrogen excretion was affected by treatment (P < 0.01) with higher breath hydrogen excretion observed after both RKB and RK evening meals compared with WWB (P < 0.01 and P = 0.04, respectively) (Fig. 3).
No difference between RK and RKB were observed. Breath hydrogen excretion in the morning at fasting and after 180 min was neither correlated with any of the appetite parameters at fasting and 180 min nor with ad libitum energy intake.

SCFA analysis and pH measurement after in vitro fermentation

All test products resulted in increased amounts of total SCFA, acetate, propionate, and butyrate after 6 h and 24 h bacterial fermentation compared to inoculum ($P < 0.001$). There was a significant effect of product on total SCFA, acetate, propionate, and butyrate after 6 h fermentation ($P < 0.001$) and on total SCFA, propionate, and butyrate after 24 h fermentation ($P < 0.001$). After 24 h fermentation total SCFA was 30% higher after RKB than RK fermentation ($P < 0.001$). In all samples pH decreased after 6 h and 24 h of fermentation, with similar values in inulin, RKB and RK. Amounts of total SCFA and pH measurements are reported in Table 2 and SCFA ratio after 24 h bacterial fermentation is shown in Fig. 4.

Changes in fecal saccharolytic bacteria after in vitro fermentation

Overall, a relatively large inter-individual variation in bacterial densities among samples inoculated with the fecal samples from the three donors was observed. Results on relative quantities of Bifidobacterium, the C. coccoides group, and Bacteroides are shown in Fig. 5 and all analyzed saccharolytic bacteria are presented in Supplemental Table S2 in the online version at doi:10.1016/j.appet.2014.05.026 of the online supplemental material. The densities of Bacteroides and the C. coccoides differed between the different products after 6 h and 24 h fermentation ($P < 0.001$), and densities of Bifidobacterium tended to differ between products after 24 h bacterial fermentation ($P = 0.001$). An effect of time was observed on the fecal community of Bacteroides after RKB and RK fermentation, on the C. coccoides group contents after RK fermentation, and on Bifidobacterium densities after inulin and RK fermentation. The effect of time on Bifidobacterium communities after RKB fermentation was also close to significant ($P = 0.003$). After 24 h fermenta-
tion, densities of Bifidobacterium were significantly higher \((P < 0.001)\) in the fecal communities fermented on inulin and RK compared with inoculum. Furthermore, Bifidobacterium content was significantly higher \((P < 0.001)\) after 24 h fermentation of RKB and RK compared with cellulose, but not different from inulin. Densities of the C. coccoides group in samples incubated with RKB were significantly reduced \((P < 0.001)\) compared with inoculum after 24 h fermentation. The relative quantities of Bacteroidetes were significantly lower \((P < 0.001)\) in fecal communities fermented on RKB and RK than in the inoculum.

PCA Bi-plot and correlations

PCA was used to reveal correlations between the presence of specific bacteria and the production of specific SCFA (acetate, propionate and butyrate) and to generate an overview of the variation in the ability of the microbial communities to ferment RKB, RK, inulin, and cellulose at 0, 6, and 24 h. Data were visualized in two dimensions using a principal component Bi-plot (Fig. 6). The two principal components (PC) explained 69.2% of the total variance between the samples. The fermentation samples were clearly differentiated by PC1, with inulin, RKB and RK after 24 h fermentation situated to the left and inoculum as well as cellulose, RKB and RK after 6 h fermentation and cellulose after 24 h to the right of the PCA Bi-plot. Samples appearing to the left were characterized by inulin for 24 h correlated with C. coccoides group and Firmicutes and with butyrate and propionate.

Spearman correlations revealed a positive correlation of Bacteroidetes and Bacteroidetes with pH \(r_s = 0.47, P < 0.01, r_s = -0.37, P < 0.01\), respectively) and a negative correlation of Bifidobacterium and Actinobacteria with pH \(r_s = -0.42, P < 0.01\) and \(r_s = -0.37, P < 0.01\), respectively.

**Discussion**

The results of the present human pilot study showed that both RKB and RK, consumed as late mixed evening meals, increased breath hydrogen excretion and decreased ad libitum energy intake at lunch at the subsequent day, although differences in subjective appetite sensation between breakfast and lunch did not reach significance. A second meal effect of wholegrain rye products on appetite sensation in comparison to WWB has been demonstrated by others. In a study by Isaksson et al. (2011), boiled rye kernel and rye kernel bread breakfasts were found to reduce hunger and increase satiety after a standardized lunch, respectively (Isaksson et al., 2011). In another study, feelings of hunger and desire to eat were reduced in the afternoon, following consumption of different rye bread breakfasts that contained either rye bran, an intermediate rye fraction, or sifted rye flour (Isaksson, Fredriksson, Andersson, Olsson, & Åman, 2009). In contrast to these findings, appetite feelings in the afternoon were not affected by a breakfast porridge made of wholegrain rye flakes, also not after three weeks of regular wholegrain consumption (Isaksson et al., 2012). This agrees with the current study that did not confirm a second meal effect on appetite sensation after wholegrain rye evening meals.

**Table 2**

| pH and total SCFA in inoculum and after 6 h and 24 h bacterial fermentation of inulin, cellulose, RKB, and RK.  
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<td>pH</td>
<td>Inoculum 6 h Fermentation</td>
<td>Inoculum 24 h Fermentation</td>
<td>Inoculum 6 h Fermentation</td>
<td>Inoculum 24 h Fermentation</td>
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<tr>
<td>Total SCFA (mmol/L)</td>
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<td>5.0 ± 0.1</td>
<td>6.9 ± 0.0</td>
<td>4.8 ± 0.1</td>
<td>5.0 ± 0.1</td>
<td>4.6 ± 0.0</td>
<td>6.2 ± 0.1</td>
<td>4.4 ± 0.1</td>
</tr>
</tbody>
</table>
|                                | 1.9 (0.4)       | 41.3 (7.7)  
|                                | 14.7 (2.1)       | 16.1 (6.1)       | 24.8 (6.7)       | 57.5 (25.9)       | 32.1 (3.8)       | 32.6 (19.7)       | 46.5 (6.3)       |

RK, wholegrain rye kernel bread; RKB, boiled rye kernels; SCFA, short-chain fatty acids (given as the sum of acetate, propionate, butyrate, iso-butyrate, valerate, and iso-valerate).

Significance compared with inoculum \((P < 0.001)\) is indicated by *.

Values are presented as unadjusted mean ± SD for pH values and median (interquartile range) for total SCFA. Values with different superscripts within row and within 6 h and 24 h fermentation, respectively, differ significantly \((P < 0.001)\).
Despite lack of overall effect on appetite sensation, we observed an effect of wholegrain rye consumption on ad libitum energy intake at the third meal. Similarly, Johansson et al. (2013) reported reduced ad libitum energy intake at lunch after a barley kernel evening meal (Johansson et al., 2013). In contrast, Isaksson et al. (2008) did not find an effect on ad libitum energy intake in the afternoon 8 h after ingestion of a rye porridge breakfast, despite decreased hunger and increased satiety ratings following a standardized lunch meal (Isaksson et al., 2008), which may be related to a smaller effect with a porridge compared with kernels or bread.

In the present study, the increased breath hydrogen levels at the next morning after both the RKB and RK test meals indicate an increased colonic fermentation. It has been hypothesized that appetite sensation may in part be regulated by the fermentation products SCFA (Cani et al., 2009), as also suggested by a previously reported positive correlation of breath hydrogen levels with satiety sensation (Nilsson et al., 2008b) and a negative correlation with ad libitum energy intake (Rosen et al., 2011) in human studies. In the current study no correlations were found, possibly because rye seems to ferment rather early and the time point of peak fermentation may thus already have passed (Isaksson et al., 2012; Nilsson et al., 2008b). However, the increased breath hydrogen levels in the morning after the RKB and RK evening meals are still suggestive of an involvement of colonic fermentation in the reduced energy intake at the ad libitum lunch, although causality has not been established.

Interestingly ad libitum energy intake was affected, despite a continuous lowering in colonic fermentation in the course of the morning, as indicated by the decreasing levels of breath hydrogen. Also the appetite ratings suggest potential effects primarily in the late-postprandial period, although differences only reached significance at a few individual time points. In line with this, Johansson et al. (2013) also observed a reduced ad libitum energy intake at lunch after a barely kernel evening meal, while breath hydrogen excretion was already decreasing (Johansson et al., 2013). Thus, it may be hypothesized that events occurring during colonic fermentation influence energy intake at later time points, even though fermentation may already subside.

The underlying mechanism connecting colonic fermentation and appetite sensation are not fully understood. It has been proposed that binding of SCFA to specific receptors located on colonic L-cells may lead to secretion of the satiety-inducing gut hormone GLP-1 (Tolhurst et al., 2012). Increased GLP-1 levels have been observed after one to ten weeks consumption of fermentable dietary fibers in both animal (Cani, Neyrinck, Maton, & Delzenne, 2005; Parnell & Reimer, 2012) and human studies (Cani et al., 2009; Piche et al., 2003). However, in the current study, the evening test meals were only consumed at a single occasion and it is therefore doubtful that GLP-1 played a considerable role. This is also supported by the lack
of an effect on GLP-1 after a one-time ingestion of 24 g inulin as observed by Tarini and Wolfer (2010). An attenuated second meal glycemic response has been observed following wholegrain consumption (Nilsson et al., 2008b). One can speculate that this may have influenced the ad libitum energy consumption at lunch in the current study, as a lower glucose response has been found to be associated with reduced ad libitum energy intake (Flint et al., 2006). However, the design of the present study does not allow any conclusions on the underlying mechanisms.

Resistant starch has been suggested to be among the main fermentable components in wholegrain. Owing to their intact structure, whole kernels are naturally high in resistant starch and this may explain the apparently higher colonic fermentation compared with their milled counterparts (Nilsson et al., 2008b). In the current study, the highest resistant starch content was expected for RK and intermediate content in RKB. Although such differences in resistant starch were confirmed by the in vitro resistant starch measurements, similar breath hydrogen levels were measured after the ingestion of RKB and RK, indicating a similar extent of colonic fermentation after the two meals. This suggests that other fermentable components in rye may determine total colonic fermentation, outweighing possible differences in resistant starch, which agrees with the similar dietary fiber content in the RK and RKB. Wholegrain rye is especially rich in fructans and arabinoxylans (Kamal-Eldin, Aman, Zhang, Bach Knudsen, & Poutanen, 2008), which both have been found to be readily fermentable as well as to affect appetite sensation in humans (Cani et al., 2009; Hartvigsen et al., 2014).

The fermentability of both RK and RK was confirmed by the in vitro fermentation, as implied by the drop in pH and increase in SCFA concentrations. However, when interpreting these data, one needs to consider that they rather provide an indication of colonic events and are less suitable for a direct comparison to the human study. Bifidobacterium is considered to be an important saccharolytic bacterial genus due to its potentially beneficial effect on health (De Vuyst & Leroy, 2011). Wholegrain products have been reported to exhibit a bifidogenic effect in vitro and in vivo (Carvalho-Wells et al., 2010; Connolly, Tuohey, & Lovegrove, 2012; Costabile et al., 2008). In the current experiment, in vitro fermentation of RKB and RK stimulated the growth of Bifidobacterium to a similar extent as inulin, which is well-known for its bifidogenic properties (De Vuyst & Leroy, 2011).

In contrast, the high availability of fermentable components did not lead to growth of Bacteroides, another dominant saccharolytic genus in the colon (Lay et al., 2005). Bacteroides decreased to the same extent after in vitro fermentation of both RKB and RK compared with initial concentrations, despite their advanced saccharolytic system (Duncan et al., 2009). This may be ascribed to the relatively high pH-sensitivity of these bacteria (Duncan et al., 2009), which is supported by a positive correlation between Bacteroides and pH in the current experiment. It needs to be considered that the in vitro fermentation was not pH adjusted and therefore yielded unphysiologically low pH values. However, it has been reported that Bacteroides growth ceases already at pH values of 5.5, which are likely to prevail during dietary fiber fermentation under physiological conditions (Duncan et al., 2009). Thus, the current data support that a high substrate availability promotes the growth of pH-resistant bacteria, such as Bifidobacterium (Duncan et al., 2009), whereas pH-sensitive bacteria may be more competitive under conditions of limited substrate availability. Concurrently, high Bacteroides levels are associated with one of the three known gut microbiota enterotypes that is associated with a typical Western diet, i.e. a diet high in fat and protein and low in dietary fibers (Wu et al., 2011). Bacteroides have also been suggested to be associated with energy intake. Results, however, are contradictory, as Bacteroides levels were found to be positively correlated with ghrelin and energy intake in rats (Parnell & Reimer, 2012) whereas they were negatively correlated with habitual energy intake in a cross-sectional study of monozygotic twins (Simões et al., 2013). Thus, the causal role of Bacteroides remains elusive.

Surprisingly, the amount of total SCFA was observed to be lower after fermentation of RK and especially RKB than after inulin, despite similar pH values. We speculate that RK and RKB fermentation may lead to a higher production of other acidic fermentation products such as lactate, which is produced by bacteria such as Bifidobacterium (De Vuyst & Leroy, 2011). Similar to SCFA, lactate is also assumed to affect appetite sensation, as lactate infusions have been found to reduce ad libitum energy intake (Schultes et al., 2012). Thus, these data suggest that the decreased ad libitum energy intake at lunch after the RKB and RK evening meals may possibly be mediated by a mechanism based on generation of both SCFA and lactate during fermentation.

Although the SCFA ratios after RKB and RK fermentation seem to resemble one another more than the profile after inulin, the butyrate concentrations were considerably lower after 24 h fermentation of RKB than RK, as was the density of the bacteria from the butyrate-producing C. coccoides group (Hayashi, Sakamoto, Kitahara, & Benno, 2006). As resistant starch fermentation is known to lead to an increased butyrate production (Topping & Clifton, 2001), higher amounts of residual starch after in vitro digestion of RK than RKB may be one explanation for these differences.

It is necessary to consider that in vitro fermentation is a model system that does not account for colonic absorption and secretion or control for pH. As the inter-individual variation in the gut microbiota is generally large (Lay et al., 2005) and only three fecal donors were used, the current in vitro fermentation data should be considered as a pilot experiment that may only give some insight into the complex colonic system (De Vuyst & Leroy, 2011). Furthermore, association of in vivo and in vitro data should only be done with caution. Finally, due to the small sample size in the human intervention study, these results should also be interpreted with care and need to be confirmed in future studies.

Conclusion

Late evening meals with RKB and RK reduced ad libitum energy intake at lunch on the subsequent day and colonic fermentation may
be a possible underlying mechanism. In vitro fermentation of RKB and RK stimulated the growth of certain saccharolytic bacteria, whereas it inhibited growth of others. More short- and long-term studies are needed in order to investigate the underlying mechanisms associating colonic fermentation of wholegrain rye and appetite sensation.

Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.appet.2014.05.026.

References


