Title: At a high dose even partially degraded beta-glucan with decreased solubility significantly reduced the glycaemic response to bread.

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Abstract

Cereal beta-glucan can reduce post-prandial glycaemic responses, which makes it an interesting ingredient to improve the health impact of bread, a staple food with a high glycaemic index (GI). Here we compare the ability of different wheat-based breads prepared with oatbran concentrate and barley flour and a Norwegian type of soft wrap (lompe) for their ability to reduce glycaemic responses in healthy adults. Both breads with the highest beta-glucan content (3.8 g per serving) significantly reduced peak blood glucose rise (PBGR), incremental area under the blood glucose curve (iAUC) and GI compared to wheat control regardless of beta-glucan M_w and solubility. At a medium dose of 1.7 g per serving breads with beta-glucan of high MW and solubility significantly lowered iAUC, but not GI or PBGR compared to white bread. In contrast to previous studies, no significant correlation between viscosity after *in vitro* digestion and any of the glycaemia variables was found. However, the amount of soluble beta-glucan per serving was inversely correlated with GI. Lompe had a similar medium GI (63) than the high dose beta-glucan breads (56 and 64). However, while "lompe" had significantly lower amounts of rapidly digestible starch, no differences in *in vitro* starch digestion were found between the different breads. Instead, increased local viscosity at the intestinal border (e.g. soluble beta-glucan interacting with the mucus layer), dilution of nutrients (higher water content and serving size) and/or reduced gastric emptying are proposed as potential explanations for the lower glycaemic responses to high dose beta-glucan breads.

1. Introduction

Glycaemic response (GR) is the post-prandial rise in blood glucose after ingestion of a food or meal containing available carbohydrate. The extent of the GR to a food does not only depend on the amount of available carbohydrate, but also on its physiological properties [1, 2]. The glycaemic index (GI) is used to compare the carbohydrate quality of different foods. The test and reference food (glucose or white bread) must contain the same amount of available carbohydrate (usually 50g). The GI expresses the incremental area under the blood glucose response curve (iAUC) for the test food in each subject as a percentage of the same subjects mean reference iAUC [3]. The GI of the food is the mean of the GI values calculated for each subject and at least 10 subjects are needed to get good estimates of the GI of a test product [4, 5]. Several health benefits including reduced risk of cardiovascular disease, metabolic syndrome and type II diabetes have been associated with low-GI diets [3, 6-8]. However, there is a lack of low GI foods and many staple foods such as bread have a high GI [9]. Nevertheless, there is a huge potential for low GI staple foods such as bread since exchanging common bread with low GI bread made with whole cereal kernels for only three weeks was enough to improve insulin sensitivity in patients with impaired glucose tolerance [10].

Even though wholegrain wheat breads contain relatively high amounts of dietary fiber (usually mostly insoluble), the GI of wholegrain wheat bread is similar to that of white wheat bread [11, 12]. Intact cereal kernels are effective in reducing glycaemia, presumably because the intact botanical structure reduces starch accessibility [13]. Intact kernels and organic acids have been used to create low GI breads [11]. However, not all consumers like breads with intact kernels or an acidic taste. Instead, soluble dietary fibers such as cereal beta-glucan can be used to produce low GI breads [11]. Many of the proposed mechanisms by which soluble dietary fibers may influence glycaemic response are related to their viscosifying properties and include delayed

gastric emptying, changes in hormonal regulation, delayed or reduced starch degradation and delayed sugar absorption [14].

Among different soluble dietary fibers, cereal beta-glucans are especially interesting, due to their high natural content (4-7 %) in the cereals barley and oat [15] and their health claims approved by the European Food Safety Authority (EFSA) on reduction of blood cholesterol levels [16, 17] and reduction of post-prandial glycemic responses [18]. However, the use of the EFSA claim "reduces post-prandial glycemic response" requires foods to contain 4 g beta-glucan per 30 g available carbohydrate. This is difficult to achieve in bread, but improvements in dry fractionation of cereal grains have resulted in an increased availability of high beta-glucan (with 10 to 30% beta-glucan content) flours from barley and oat, which may facilitate the production of foods that qualify for EFSA health claims thus inspiring the food industry to produce products with high beta-glucan contents

However, clinical studies have shown that beta-glucan molecular weight (MW), solubility and viscosity (after *in vitro* digestion) are important parameters influencing the glycaemic response to e.g. muffins, extruded breakfast cereals and granola with equal beta-glucan contents [19-22]. To ensure optimal reduction of GR or facilitate similar effects at lower doses, beta-glucan MW and solubility in food products must be optimized. During bread production, beta-glucans are degraded by endogenous enzymes, but strategies that minimize this reduction have been developed and employed for barley bread [23]. Increasing the understanding of the effect of beta-glucan amount, MW, and solubility and the mechanisms by which beta-glucan may influence GR to bread along with strategies of how the physicochemical properties of beta-glucan may be controlled during bread production will help the food industry to develop low GI breads. To increase the consumption of low GI foods, the selection of staple foods with low GI needs to be improved. Common Nordic food items have long been recognized to lack reliable GI data [24]. Increasing the number of food items with a valid GI may potentially identify good low GI

stable food candidates. A Norwegian type of unleavened potato-cereal flour, tortilla-like, soft wrap known in Norway as “lompe” is an interesting candidate due to its high content of cooked and cooled potatoes. The GI of “lompe” has (to our knowledge) never been determined, and “lompe” was therefore included in the present study along with breads containing different amounts of beta-glucan (fulfilling the EFSA criterion and at lower doses) varying in MW and solubility. The foods were tested *in vivo* for their ability to reduce post-prandial glycaemic responses in healthy humans. The clinical trial was supplemented with i*n vitro* digestion experiments studying the solubility of beta-glucan during digestion, the MW of the dissolved beta-glucan molecules, their contribution to viscosity during digestion and their effect on starch digestibility. We use this information to discuss and give a glimpse into the potential mechanisms by which beta-glucans in bread may elicit their hypoglycaemic effect.

2. Material and Methods

Ingredients

Commercial wheat flour of high protein strength was obtained from Lantmännen (Lantmännen Cerealia, Oslo, Norway). Barley flour was produced on a laboratory hammer mill (Retsch, Model ZM100, Retsch GmbH, Haan, Germany) with a 0.5 mm mesh from barley flakes prepared from de-hulled Olve (a Norwegian barley variety) micronized and flaked by Lantmännen Cerealia (Moss, Norway). An oatbran concentrate (OBC) containing 14 g beta-glucan per 100 g was obtained from Swedish oat fiber (SweOat bran BG14 bakery, Swedish oat fiber, Bua, Sweden). Dry yeast from Idun (Idun, Oslo, Norway) and vegetable fat and oil from A/S Pals (A/S Pals, Oslo, Norway) were used to prepared the experimental breads.

Experimental foods

The 5 different test breads comprised a wheat control and four breads with different beta-glucan contents and processing. The breads were designed to fulfill the criteria of the EFSA health claims on reduction of postprandial blood glucose rise [18] or reduction of LDL-cholesterol [16], which require beta-glucan doses of 4 g per 30 g available carbohydrate or 1 g per serving respectively. The formulation and processing of the breads is summarized in table 1 and described in detail below.

Table 1: Overview of the preparation and processing of the different test breads.

Bread	Fulfills the criteria of the EFSA health claim on		Flour	Processing
	Reduction of post-prandial blood glucose rise [a]	Reduction of LDL-cholesterol [b]		
degradedOBCB	√	√	50% OBC + 50% wheat	Long process
optimalOBCB	√	√	50% OBC + 50% wheat	Separate doughs
lowOBCB	x	√	25% OBC + 75% wheat	Separate doughs
Barley bread	x	√	40% barley + 60% wheat	Separate doughs
Wheat control	x	x	100% wheat	Standard

[a] Requires the product to contain at least 4 g beta-glucan per 30 g available carbohydrate
[b] Requires the product to contain at least 1 g beta-glucan per serving/portion and the package to give information about the required total daily dose of 3 g beta-glucan.

One of the breads (degradedOBCB) was prepared by a process designed to induce degradation of beta-glucan during bread production. This was achieved by mixing all ingredients together and applying an unusually long proofing time of 5h, which gives the beta-glucan degrading enzymes in the wheat flour enough time to depolymerize the beta-glucan in OBC [23]. The barley bread was produced by a previously optimized baking procedure, which minimizes beta-glucan molecular weight reduction [23]. The procedure involves the development of a pure wheat flour dough, which is then fermented for 1h before the barley flour and additional water is added. The same approach (separate doughs) was used to prepare one of the high dose OBC breads (optimalOBCB) and the lowOBCB. All breads contained 1 g dry yeast, 1.5 g NaCl and 1 g fat per 100 g flour. All wheat flour doughs were prepared with 58.4% water (on flour basis) in a spiral mixer (Diosna sp12, Diosna, Osnabrück, Germany) for 2 min at low and 6 min at high speed. Dough temperature after mixing was 27 ± 1 °C. The wheat flour doughs were fermented for 1h at 27°C and 70% RH in a fermentation cabinet (Lillinord AS, Odder, Denmark). The OBC was pre-hydrated for 1h at RT with 200 g water per 100 g OBC. The water addition to OBC was optimized empirically to achieve acceptable dough handling properties and bread quality. The pre-hydrated OBC or barley flour + water (103% based on barley flour weight)

was then incorporated into the fermented wheat flour dough for 2 min at low speed (Diosna sp12) for optimalOBCB, lowOBCB and barley bread respectively. Doughs were divided into pieces of 243 g (optimalOBCB and degraded OBCB), 162.8 g (lowOBCB), 128.6 g (barley bread) and 110 g (white bread), corresponding to 50g available carbohydrate, molded, placed in small steel pans and proved at 30°C and 70%RH for 30 min (barley bread), 45 min (white bread, lowOBCB, optimalOBCB) or 5h (degradedOBCB). Breads were baked in an rotating hearth oven (Revent type 626 G EL IAC, Revent international, Väsby, Sweden) for 20 min. Immediately after the loaves were put into the oven, the temperature was reduced from 240 to 220 °C and steam (0.5 L water) was injected during the first 10 sec. One hour after baking weight (scale) and volume (TexVol BVM –L 370, TexVol insturments AB, Viken, Sweden) of the breads was determined.

In addition to the five different breads, commercial lompe made from potato (pre-cooked and cooled) and spelt flour (a brief description of the baking process is given in [25]) was obtained from Buer (Speltlompe, Buer AS, Askim, Norway) and included in the clinical study. All six test products (five breads and one "lompe" were frozen in a rapid freezer (Blast freezer, Lillnord, Odder, Denmark) stored and shipped frozen prior to consumption in the clinical trial and analysis.

Chemical composition

The contents of moisture [26], protein [27], fat [28], total dietary fiber [29], total beta-glucan [30] and ash [31] as well as available and resistant starch [32] were determined in the foods using standard methods. More details on the analytical methods can be found in the supplementary. Total energy content per serving was calculated from the nutrient composition according to EU Council Directive 1169/2011 and available carbohydrate was calculated as described by Brouns et al. [4].

Physicochemical analysis of beta-glucan in breads

To determine the physicochemical characteristics of beta-glucan in the breads under physiological conditions, all test foods were subjected to an *in vitro* digestion procedure based on the Infogest protocol [33]. A detailed description of the experimental procedure as applied in our lab has been published earlier [34]. After digestion, samples were centrifuged and the rheological properties of the supernatants were characterized using a Physica MCR 301 rheometer fitted with a double gap (DG26.7) geometry. Apparent viscosity was measured at 37˚C in a shear rate range of 0.5 to 500 s^{-1} with seven measurement points per decade. The measurement point duration ranged from 20 to 0.1 s during the forward ramp and 0.1 to 20 s during the backward ramp. The Cross-equation was used to calculate the zero shear viscosity of each solution using data from the forward ramp [35]. Low viscous samples that did not show shear thinning were measured at a constant shear rate of 10 s^{-1}. All extracts were incubated with 5 U Lichenase (endo-1,3(4)-β-glucanase, E-LICHN, Megazyme) after which viscosity was determined again. This approach enables the determination of the viscosity contribution of the solubilized beta-glucan. Further details on the experimental procedure can be found elsewhere [34]. Beta-glucan concentration and weight average molecular weight (M_w) were determined in the extracts after digestion using HPSEC with post column calcofluor detection. Samples were filtered (0.8µm syringe filter, Millipore) before injection of 50µL into an HPLC system consisting of two pumps (Dionex UltiMate 3000), an auto injector (Dionex UltiMate 3000), a pre-column (Tosoh PWXL), two serially connected columns (Tosoh TSK-gel G5000 PWXL and G6000PWXL, maintained at 40°C) and a fluorescence detector (Shimadzu RF-10A, Shimadzu Europa, Duisburg, Germany). A flow rate of 0.5mL/min was used to deliver the eluent (50mM Na_2SO_4), while Calcofluor (Megazyme) solution (25mg/L in 0.1M tris(hydroxymethyl)aminomethane) was delivered post-column through a T-valve at a flow rate of 0.25 mL/min. Fluorescence detection of the formed Calcofluor/β-glucan complexes occurred

at λ_{ex} = 415nm and λ_{em} = 445nm. A calibration curve for β-glucan MW was constructed with in house β-glucan MW standards and standards purchased from Megazyme with peak MW from 31600 to 2418000. A proprietary third order polynomial regression (PSS poly 3) was used for peak position calibration using PSS WinGPC Unichrome software (PSS Polymer Standard Service, Mainz, Germany). Different dilutions of a standard beta-glucan solution (245kDa cereal beta-glucan MW standard, Megazyme) were injected into the HPSEC system. A linear regression was fitted to the area under the chromatographic peaks and the beta-glucan concentration of the standards for each sequence run ($R^2 > 0.99$). This regression was used to calculate the beta-glucan concentrations in the different extracts from the area under the chromatographic peak. Beta-glucan concentrations in the extracts were used to calculate beta-glucan solubility under physiological conditions.

In vitro **starch digestibility**

As a potential *in vitro* predictor of glycemic response the amount of rapidly digestible starch (RDS) and the kinetics of glucose release from the test products were determined [36-38]. The *in vitro* digestion protocol employed for this purpose was based on the Infogest model [33] and a more specialized method for starch digestibility published by Monro et al.[38] . The different breads and lompe were thawed overnight, chewed until the urge to swallow and then expectorated. The expectorated material was thoroughly mixed and 2 g aliquots were weighed into 50mL centrifuge tubes in duplicates for each time point. The rest of the expectorated material was used to determine the moisture content according to AACC 44-15A. The samples were first subjected to a simulated gastric digestion at pH 3 and 37°C for 1h. Buffer and enzyme (pepsin) addition were as earlier described [34] and according to the Infogest protocol [33]. After the gastric phase, 4mL pre-warmed (37°C) 0.1M Na-maleate buffer pH 6 with 0.2% Na azide and 1mM $CaCl_2$ containing 200 U/mL pancreatin (based on trypsin activity, P1750 from porcine pancreas, Sigma-Aldrich, St Louis, US) were added to each tube, together with 50µL

amyloglucosidase (3300 U/mL on soluble starch, Megazyme) and pH was adjusted to 6 by adding pre-determined amounts of 1M NaOH. Tubes were vortex mixed and placed horizontally in a shaking incubater (Innova 40, Incubator Shaker Series, New Brunswick Scientific, Edison, New jersey, US) at 175rpm and 37°C. After 120 min incubation, the two remaining tubes were vortex mixed vigorously before incubating further for a total of 180 min. The reaction was stopped after 0, 10, 20, 40, 60, 120 and 180 min by adding 32 mL ethanol to each of the two tubes per time point. Tubes were centrifuged and the supernatants were diluted with water (1:10) before aliquots of 100µL were mixed with 500µL 200mM Na acetate buffer pH 5.2 containing 33 U/mL amyloglucosidase (Megazyme). After 20 min incubation at 50°C, released glucose was measured spectrophotometrically using a glucose oxidase assay (Megazyme). Based on the moisture content of the chewed expectorated material and the chemical composition of the test foods, the total amount of starch per 2 g of chewed sample was calculated and compared to the total amount of released glucose (calculated as starch) to give the amount of digested starch either as % of total starch or in g per serving for each time point.

Clinical trial

The clinical trial was based on international recommendations for glycaemic index testing [4]. At the screening session fifteen healthy subjects were recruited after meeting the inclusion and exclusion criteria. The inclusion criteria were age (18-65 years), BMI (18-27 kg/m2), gender (both male or female) and self-diagnosis as healthy (medical questionnaire), while subjects with a history of diabetes were excluded. Before each session subjects that had consumed anything apart from water 12h prior to the test, were excluded from the study. Informed written consent was obtained from all volunteers before study start. The study was conducted according to the guidelines of the Declaration of Helsinki and the study design was approved by the National Research Ethics Service, West Kent Research Ethics Committee, Aylesford, UK

(09/H1101/59). All clinical testing was conducted at Leatherhead Research Ltd, UK within a three month period between October-December 2015

The mean age of the subjects was 44.76 years (SEM 3.69) with a mean BMI of 24.26 (SEM 0.44) kg/m^2. One subject did not consume one of the breads, another subject tested the reference glucose only twice rather than three times. Otherwise, all 14 subjects completed all nine visits. The study was a randomized block design with repeated measures with each subject testing the six different breads once and the glucose control three times (in the beginning, middle and end of the study). Mean values of the three glucose reference tests of each subject were used for statistical analysis.

Tests were conducted in the morning after an at least 12h overnight fast. Subjects were instructed to avoid strenuous exercise, smoking and alcohol consumption the evening before a test and consume a similar carbohydrate-based evening meal before each test session. There was at least a 48h wash out period between the tests. Subjects had to consume the test products within 15 minutes with 250 mL of water. Since it was impossible for some subjects to consume the initial portion size (corresponding to 50 g available carbohydrate) of the high beta-glucan breads within 15 minutes, the portion size was decreased to contain 25 g available carbohydrate for all breads and the glucose reference. Finger prick capillary blood samples were taken at 0, 15, 30, 45, 60, 90, 120 and 180 min. Blood samples were collected into small tubes containing lithium-heparin and centrifuged at 3000 rpm for 10 min to separate plasma. The plasma samples were then analysed for glucose by an YSI 2300 Stat Plus Glucose and Lactate Analyser (sensitivity 0-50 mmol/L and margin of error +/- 2%).

Calculations and statistical analysis

The incremental area under the glucose response curve (iAUC) above fasting baseline was calculated from 0-120 min using the standard trapezoid geometric method as previously

described [39]. Peak blood glucose rise (PBGR) was calculated as the differences of each subject`s peak and fasting glucose values. The GI was calculated by expressing the iAUC for the test food in each subject as a percentage of the same subjects mean reference (glucose) iAUC. The GI of the food was the mean of the GI values calculated for each subject. The mean and coefficient of variation (CV = 100xSD/mean) of within-individual iAUC values for repeated measures of the reference food (25g glucose) was calculated for each subject. The mean CV for the subject group was with 21.1 below the upper recommended threshold of 30 [5]. Individual values of iAUC or GI greater than the mean plus 2 times standard deviation (SD) were considered outliers and removed from the final results as previously recommended [5]. The influence of this outlier removal is discussed in the results section.

All statistical analysis were performed using Minitab version 18. Statistical differences between mean iAUC, GI and peak blood glucose rise (PBGR) for each test food were assessed by repeated measures ANOVA using a general linear model with test food (fixed) and subject (random) as factors. Comparisons between test foods were made with the *post hoc* Tukey pairwise comparison test at a confidence interval of 95%. For the five different breads (not lompe), linear regression analysis and Pearson correlation were used to examine the relationship between the glycaemia variables (iAUC, GI and PBGR) and different bread characteristics (beta-glucan: Mw, concentration after *in vitro* digestion (c), viscosity after *in vitro* digestion, total amount, and amount of soluble beta-glucan, Mw x c and Mw x amount of soluble beta-glucan in linear and log10 scale and serving size). A value of $p < 0.05$ was considered to be statistically significant.

3. Results and Discussion

Postprandial blood glucose response

The blood glucose rise after ingestion of the test foods differed substantially from the blood glucose rise of the glucose reference (Figure 1 and Table 2). All test foods elicited a significantly lower peak blood glucose rise (PBGR) than the glucose reference (Table 2). For barley bread and lompe, outlier removal changed the PBGR from 2.82 to 2.68 (outlier 4.86) and from 2.19 to 2.04 (outlier 4.34), respectively. Among the test foods, white bread had the highest PBGR, followed by barley bread and lowOBCB. The PBGR elicited by optimalOBCB, degradedOBCB and lompe was significantly lower than for white bread (Table 2), although there was no significant difference in PBGR between these 3 types of bread (Table 2)

For lowOBCB, degradedOBCB and optimalOBCB, outlier removal changed the average iAUCs from 126.7 to 104.5 (outliers 270 and 272), 114 to 107.1 (outlier 211) and 117.3 to 106.8 (outlier 243), respectively. The iAUCs elicited by the different test foods were lower than for the glucose control (Table 2). However, for white bread and barley bread, this difference was not statistically significant. Compared to white bread, all test foods, except barley bread, resulted in a significantly lower iAUC (Table 2). For lowOBCB this difference was only statistically significant after the removal of outliers.

For white bread, lowOBCB, optimalOBCB and barley bread, outlier removal changed the GI estimates from 94.3 to 84.1 (outlier 237), 68.6 to 64.9 (outlier 121), 60.9 to 56.8 (outlier 109) and 77.1 to 71.8 (outlier 150), respectively. However, the removal of outliers did not change the differences between GI values significantly (Table 2). The GI value for white bread of 84.1 was relatively high compared to mean GI values for white bread (72.5 and 75) obtained by an inter-laboratory study or published in the international table of GI and GL [40, 41]. However, the published mean GI values (72.5 and 75) are for shop bought white bread. Industrially produced white wheat bread normally contains different additives, such as the emulsifier diacetyl tartaric

acid esters of monoglycerides (DATEM). DATEM slows down staling by interfering with starch retrogradation [42] and has been shown to reduce the GI of white bread [11, 42]. In comparison, a GI of 95 was reported for French baguette produced without additives [12]. Furthermore, specific loaf volume influences the GI of white bread [43], which further complicates the direct comparison of GI values. Nevertheless, a white bread produced without additives and with a similar specific volume (3.17 mL/g) than the white bread in our study (3.6 mL/g) also showed a similar GI of 86 [43].

The barley bread had a GI of 72, which was lower than for white bread with a GI of 84. However, the difference was not statistically significant, and the GI of the barley bread was still in the range of high GI foods (> 70). All three breads with OBC and lompe had GI in the medium range (55-70). However, the difference in GI for lowOBCB and white bread was not statistically significant.

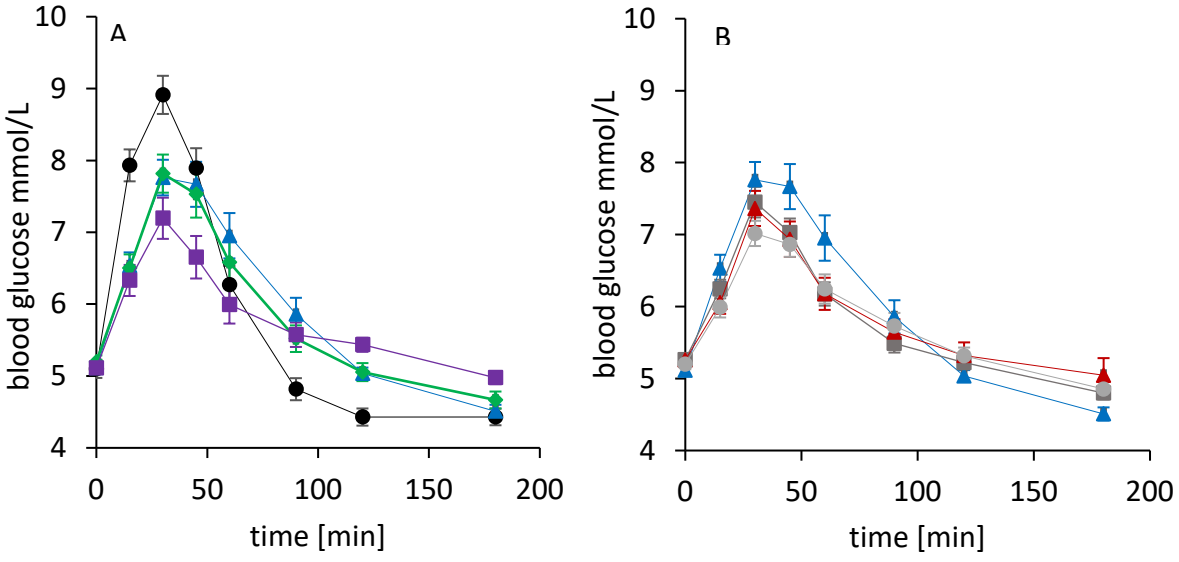

Figure 1: Changes in blood glucose with time within 2h postprandial. Values are mean +/- SEM. A: glucose reference (black dots), white bread (blue triangles), barley bread (green diamonds), lompe (purple squares). B: white bread (blue triangles), lowOBCB (dark grey squares), degradedOBCB (red triangles), optimalOBCB (light grey dots).

Table 2: Postprandial blood glucose response[a]

Food	PBGR (mmol/L)[b]	iAUC (mmol x min/L)	GI (% of control)
Control (glucose)	3.94 ± 0.24 a	180.4 ± 14.9 a	100
White bread	2.93 ± 0.27 b	165.8 ± 20.4 a	84 ± 7 a
Barley bread	2.68 ± 0.26 bc	139.9 ± 18.0 ab	72 ± 6 ab
lowOBCB	2.51 ± 0.25 bcd	104.4 ± 9.3 b	65 ± 4 ab
degradedOBCB	2.28 ± 0.21 cd	107.1 ± 10.3 b	64 ± 5 b
optimalOBCB	2.11 ± 0.19 d	106.8 ± 13.9 b	57 ± 4 b
Lompe	2.04 ± 0.27 d	113.4 ± 16.4 b	63 ± 6 b

[a] values are mean values ± SEM for all subjects after outlier correction (n= 14-12 ; values higher than mean + 2 times SD were removed). Values not followed by the same letters in columns were significantly different at $p < 0.05$.
[b] Peak blood glucose rise: difference between peak and fasting blood glucose

Effect of protein and fat

Since fat and protein can influence the GR to a test product [3, 44], the macronutrient composition of different test products is often standardized for example by adding egg white powder to equalize the protein content [22]. In the present study, we kept the ingredients as simple as possible and exchanged wheat flour for barley flour or OBC without any further adjustment of macronutrient composition. Due to the higher content of fat and protein in OBC compared to wheat flour, the breads prepared with OBC contained slightly higher amounts of protein (up to 4.2 g difference per serving) and fat (up to 2.9 g difference per serving) compared to the white bread control (Table 3). However, the differences in fat and protein are probably too small to influence GR significantly as 12.5 g protein (from tuna) and 11.1 g fat (from butter) added to white bread (50 g available carbohydrates) did not show any significant effect on GR in a previous study [45].

Table 3: Nutrient content and composition of test foods[a]

	Specific volume (mL/g)	Serving size (g)	Amount available carbohydrate[b]	Total dietary fiber	Resistant starch	Beta-glucan
White bread	3.6	53	26.0	1.4	0.25	0.1
Barley bread	2.3	59	26.0	2.3	0.34	0.8
lowOBCB	2.8	73	26.4	4.1	0.46	1.7
degradedOBCB	nd	101	26.2	8.0	0.47	3.8
optimalOBCB	2.1	102	26.3	7.6	0.46	3.8
Lompe	nd	70	26.2	2.8	0.68	0.1

	Protein	Fat	Ash	Water	Energy (kcal)	Energy density (kcal/g)
White bread	4.9	1.2	0.70	18.8	128	2.4
Barley bread	4.7	1.2	0.79	23.7	129	2.2
lowOBCB	6.6	2.3	1.17	32.8	152	2.1
degradedOBCB	9.1	4.1	1.84	51.0	185	1.8
optimalOBCB	8.7	3.7	1.75	53.3	180	1.8
Lompe	4.2	0.6	1.12	34.4	125	1.8

[a] Data are in g per serving if not otherwise stated

[b] Available carbohydrate was calculated from the measured amount of available starch using a conversion factor of 1.1 [4]

Beta-glucan dose and physicochemical properties

The different test foods were subjected to an *in vitro* digestion procedure and the beta-glucan M_w, solubility and contribution to viscosity (viscosity difference before and after the addition of lichenase) was measured in the extracts (Table 4). As expected, the long proving time of the degradedOBCB resulted in degradation of the beta-glucan by endogenous flour enzymes [23], while the shorter processes used for optimalOBCB, lowOBCB and barley bread better retained the beta-glucan M_w. The beta-glucan M_w was highest for optimalOBCB (592 kDa), followed by lowOBCB (421 kDa) and barley bread (376 kDa) and lowest for degradedOBCB (282 kDa). Interestingly, not only the beta-glucan M_w, but also the extractability of beta-glucan varied considerably between the breads. The long bread making process of degradedOBCB did not only result in a lower M_w, but also a much lower fraction of the beta-glucan in the product was solubilized during the *in vitro* digestion (Table 4). This reduced solubility is quite interesting from a technological point of view. Fermentation of the dough (with or without yeast), has

previously been reported to decrease β-glucan extractability in a time dependent manner [46-48] which has been attributed to the formation of un-extractable β-glucan aggregates [48]. The amount of soluble beta-glucan per serving was consequently considerably lower for degradedOBCB than for optimalOBCB even though the two breads contained the same amount of total beta-glucan. The viscosity of the extracts varied among the breads, but was generally quite low (Table 4). Only the optimalOBCB resulted in extract viscosities above 10mPas (10.6 mPas). OptimalOBCB was therefore the only bread that located in the region above coil overlap in a double logarithmic plot of extract viscosity against the product of beta-glucan M_w and concentration (Figure 2). As described in previous work, coil overlap has been suggested as a criteria for predicting significant *in vivo* effects on the reduction of postprandial blood glucose levels [34]. However, despite their lower viscosities after *in vitro* digestion, also degradedOBCB significantly reduced GI, iAUC and PBGR compared to the white bread control (Table 2).

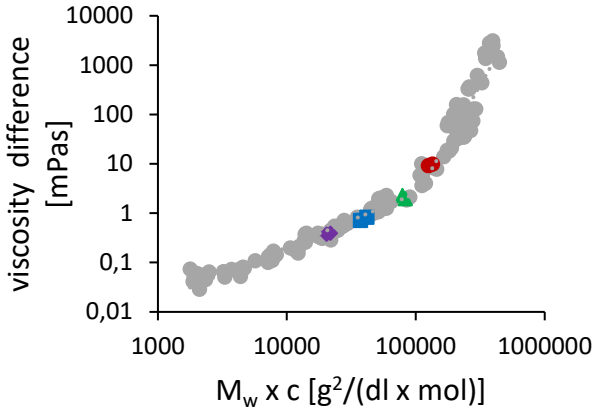

Figure 2: Double logarithmic plot of viscosity difference (before and after lichenase) against the product of beta-glucan concentration and weight average molecular weight of extracts after *in vitro* digestion. Grey circles represent data from a previous study [34]. Data from the present study are in colour: optimalOBCB (red circles), degradedOBCB (blue squares), lowOBCB (green triangles) and barley bread (purple diamonds).

Unlike many previous studies on oat bran muffins [20, 22], extruded cereals [19] and baked granola [21] treated to vary in beta-glucan solubility or MW, no significant correlations between viscosity or $\log_{10}$ viscosity and any of the glycaemia variables (GI, iAUC, PBGR) was found. Many of

the aforementioned studies compared the effect of varying beta-glucan characteristics at the same, usually high, beta-glucan dose, while our study had a broad range of different beta-glucan doses (from 1 to 4.8 g per 30 g available carbohydrate), but with relatively small differences in viscosity. The absence of a significant correlation between viscosity after *in vitro* digestion and the glycaemic response to the different breads found in our study may nevertheless point towards a less dominant role of bulk viscosity, for realistic food products, as previously suggested [49]. This is in agreement with recent suggestions, that the viscosity increase that can be expected in the intestinal lumen after the consumption of foods rich in soluble dietary fiber is unlikely to be high enough to substantially delay the diffusion of glucose [14]. However, the physicochemical properties of beta-glucan still seem to be important for its ability to attenuate glycaemic response. The amount of soluble beta-glucan per serving gave a better correlation (Pearson correlation coefficient: -0.956, p =0.011) with GI than the total amount of beta-glucan per serving (-0.884, p=0.046). Beta-glucan M_w was negatively correlated with GI (-0.883, p=0.047) and $\log_{10}$ (M_w x amount of soluble beta-glucan per serving) gave the best correlation with GI (-0.959, p=0.01) among all the different tested variables of beta-glucan physicochemical characteristics. The relationships between amount of total and soluble beta-glucan per serving, beta-glucan M_w and $\log_{10}$ (M_w x amount of soluble beta-glucan per serving) and GI are also visualized as linear regressions in figure 3. It should, however, be noted that the presented correlations and regressions are based on five observations each and therefore only can give indications.

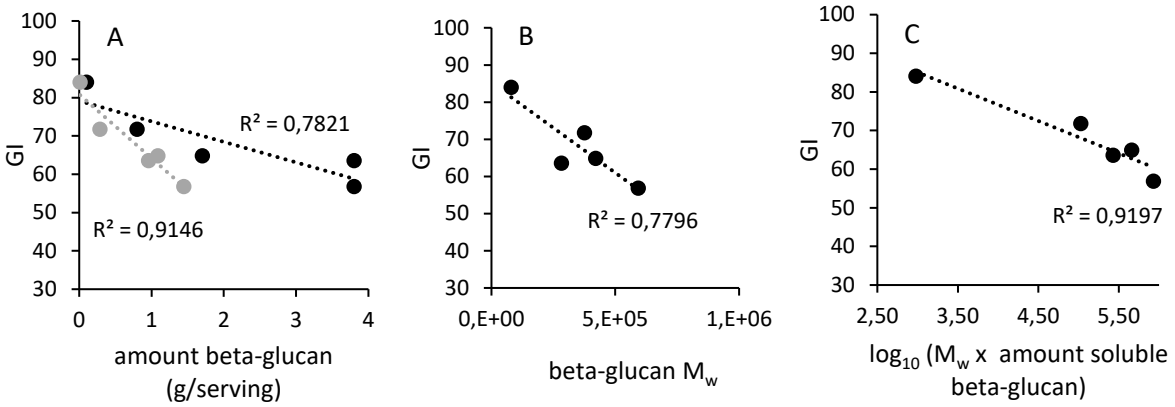

Figure 3: Correlation between GI and A: amount beta-glucan per serving (black: total beta-glucan; grey: soluble beta-glucan), B: beta-glucan M_w, C: log10 (M_w x amount soluble beta-glucan per serving).

Beta-glucan has been reported to interact with the intestinal mucus layer, thereby increasing its barrier function to lipid digestion products [50]. If such an interaction may also play a role for the diffusion of starch digestion products remains to be seen. However, it seems plausible that only the soluble/extractable fraction of beta-glucan in a food product interacts with the mucus layer, which would explain the dominant effect of soluble beta-glucan per serving for our bread products.

Interaction of beta-glucan with the mucus layer may require coil overlap, which means that the dissolved beta-glucan molecules get entangled (with each other or with other macromolecules) due to their size (MW) and concentration. The occurrence of coil overlap after *in vitro* digestion has been previously shown to correlate well with the ability of different beta-glucan containing food products to reduce glycaemic responses [34]. However, the concentration of beta-glucan at the mucus layer may be different than in the lumen. Additionally, the digestive system may adjust the volume of the meal and equilibrate viscosity by increasing the secretion of gastric fluids [51]. Interestingly, increasing the viscosity of a glucose and beta-glucan solution by reducing the solution volume had no effect on the glycaemic response, while increased viscosities brought about by higher beta-glucan MW or dose clearly reduced the glycaemic response [52].

Despite the tendencies seen in our study and the numerous studies demonstrating the importance of beta-glucan solubility and MW for their reduction of glycaemic responses [19-21, 53], there was no significant difference in iAUC, GI or PBGR between the optimalOBCB and the degradedOBCB (Table 2). Even though the latter had a significantly lower beta-glucan M_w and solubility (Table 4). Both breads were formulated to fulfill the EFSA criteria for the health claim on lowering of post-prandial glycaemic response of 4 g beta-glucan per 30 g available carbohydrate. This is a very high dose of beta-glucan, which is difficult to achieve in bread and requires the use of special milling fractions with elevated levels of beta-glucan instead of regular oat or barley flour. The resulting doughs have a very high water binding capacity and the serving size of the two high dose OBC breads was twice as much as for the white bread (Table 3). In fact, at 50 g available carbohydrate, the serving size was too big to be consumed within 15min and all the test foods were therefore downscaled to 25 g available carbohydrate. Portion size was the variable which correlated best with PBGR (Pearson correlation coefficient: -0.963, p = 0.008). The high portion size of optimalOBCB and degradedOBCB may therefore have resulted in a slower appearance of starch into the small intestine, since boluses from these two breads released from the stomach would contain less starch compared to an equal bolus of white bread.

Table 4: Physicochemical properties of test foods [a]

	Soluble beta-glucan in % of total	Beta-glucan M_w (kDa)	Viscosity of extract (mPas)	Amount soluble beta-glucan per serving (g) [b]	Amount beta-glucan (g) per 30 g available carbohydrate[c]	RDS in % of total starch
White bread		80 ± 7	1.2 ± 0.01	0.01	0.1	66.5 ± 2.5
Barley bread	35.5 ± 3.3	376 ± 16	1.6 ± 0.01	0.27	1.0	60.5 ± 1.3
lowOBCB	63.9 ± 1.1	421 ± 23	3.2 ± 0.3	1.06	2.1	60.9 ± 7.1
degradedOBCB	25.2 ± 0.1	282 ± 23	1.8 ± 0.1	0.96	4.8	68.0 ± 3.8
optimalOBCB	38.4 ± 1.7	592 ± 5	10.6 ± 0.5	1.46	4.8	62.4 ± 5.2
Lompe	nd	nd	nd	nd	0.1	46.3 ± 2.3

[a] values are averages ± standard deviations if not otherwise stated.
[b] all standard deviations below 0.006
[c] all standard deviations below 0.02

In vitro starch digestibility

The biggest difference in the rate and extent of starch digestion was seen between lompe and the bread products, while there were only minor differences among the different breads (data not shown). The content of rapidly digestible starch (RDS), which is the proportion of starch digested during the first 20 min of the *in vitro* digestion, was similar among the breads and ranged from 60.5 to 68%, while lompe had a RDS content of 46.3% (Table 4). The rate of *in vitro* starch digestibility in cereal products has been shown to correlate with the GI of the products [2]. The lower glycaemic response to lompe compared to white bread seen in this study might therefore be due to the low content of RDS in this product. The difference in glycaemic response between white bread and the breads containing OBC observed in this study can, however, not be explained by any difference in starch digestibility. This is in contrast to findings for baked oat granola, where high beta-glucan MW and high beta-glucan to starch ratios resulted in increasingly reduced levels of RDS alongside with a lower PBGR and iAUC [21]. Among others, viscosity mediated reduced enzymatic accessibility of starch and reduced availability of water for starch gelatinization and hydrolysis have been proposed as potential mechanisms by which soluble dietary fibers such as beta-glucan may reduce starch digestibility [14, 54]. The water content of the breads prepared with OBC in our study was very high (45 to 52%), while the baked granola had a water content of 40% [21], which may explain the absence of any effect on *in vitro* starch digestibility with increased amounts and M_w of beta-glucan in the breads.

Conclusions and future perspective

At the high dose of 4 g beta-glucan per 30 g available carbohydrate, even breads with process-induced reductions of beta-glucan M_w and solubility, significantly lowered PBGR, iAUC and GI compared to white bread. This might be positive, as physicochemical properties of beta-glucan are not included in the EFSA health claim definition on post-prandial blood glucose.

However, the high dose that is required is very difficult to achieve in bread, which limits the use of the claim. Here we show that nearly the same effect could be achieved with half the beta-glucan dose if the process is optimized to maximize beta-glucan MW and solubility. There was no significant correlation between the viscosity after *in vitro* digestion and any of the *in vivo* glycaemia variables. *In vitro* digestion can nevertheless give useful information on the potential of beta-glucan containing food products to reduce post-prandial glycaemic responses for example by giving information on the solubility of beta-glucan under physiological conditions as we found a significant inverse correlation between the total amount of soluble beta-glucan per serving and GI. Further studies are needed to elucidate the mechanisms of action of cereal beta-glucan, which include nutrient dilution, reduced gastric emptying, reduced starch digestibility and reduced diffusion of starch degradation products due to locally increased viscosity e.g. at the mucus layer. Apart from beta-glucan dose, MW and solubility, also the food matrix (for example the water content) may influence the efficacy and mechanism of action of beta-glucan containing food products. More information is needed before "cut off" values for beta-glucan MW and solubility that may ensure significant effects at lower doses than the current claim can be defined. Nevertheless, a future definition of such "cut off" values might help to ensure the efficacy of products bearing the claim and at the same time enable a reduction of the required dose, thereby increasing the number of food products bearing it. Typical low GI breads are often pumpernickel style breads with whole kernels or breads with high levels of organic acids, which not all consumers like. Breads containing high enough amounts of cereal beta-glucans with the right physicochemical properties or the Norwegian "lompe" may therefore be good alternatives for filling the low GI bread gap.

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Conflicts of Interest

There are no conflicts of interest to declare.

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