Jenni Lappi

Effects of Wholegrain Foods and Grain Fibre in Intestinal Tract in Relation to Glucose Metabolism

With an Emphasis on Wheat and Rye Bread Effects

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JENNI LAPPI

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ABSTRACT

Consumption of wholegrain foods is universally recommended, and wholegrain foods of wheat and rye are a good source of dietary fibre. Natural grain fibre complex including undigestable carbohydrates and bioactive compounds may have synergetic characteristics which are different from the features of isolated fibre fractions. Although prospective cohort studies show wholegrain foods to decrease the risk of type 2 diabetes, intervention studies have inconsistent effects of wholegrain and foods rich in grain fibre on factors of glucose metabolism such as insulin sensitivity and first-phase insulin secretion. It is also not known whether large-intestinal effects have a role in mediating glucose metabolism.

The aim of this work was to investigate the effects of wholegrain and grain-fibre-rich wheat and rye foods, especially breads, on the intestinal tract influencing glucose metabolism in healthy subjects and in subjects with the metabolic syndrome. Short-term effects of bread type on glucose and insulin responses and absorption of phenolic acids and their gut-derived metabolites from bioprocessed rye were investigated in two postprandial trials. In addition, two interventions were carried out to study the effects on longer-term glucose metabolism, and on large-intestinal phenomena such as the composition of the intestinal microbiota and plasma concentration of fermentation-derived short chain fatty acids.

Intake of wholegrain rye bread and sourdough-fermented wholemeal wheat bread reduced postprandial glucose and/or insulin responses. However, a 12-week consumption of these breads and other wholegrain foods did not improve insulin sensitivity or first-phase insulin secretion measured intravenously in subjects with the metabolic syndrome. When measured orally in a standardized meal test in healthy subjects, similar glucose and insulin responses after a four-week consumption of wholegrain rye bread were achieved with that of white wheat bread enriched with bioprocessed rye bran. Small-intestinal absorption of ferulic acid from rye bran was increased through bioprocessing technology without effects on glucose metabolism. Regarding large-intestinal phenomena, long-term consumption of rye-containing breads increased postprandial plasma concentrations of propionate and butyrate after a single low-fibre meal.

In conclusion, this thesis indicated that effects of wholegrain and high-fibre breads on glucose metabolism are mediated via short-term postprandial glucose and insulin responses, rather than via the studied effects in the large intestine. In future nutritional interventions, postprandial responses to a standardized meal test should be utilized in addition to fasting measurements of glucose metabolism and plasma concentrations of short chain fatty acids.

National Library of Medicine Classification:
Medical Subject Headings: Cereals; Bread; Secale cereale; Dietary fibre; Glucose metabolism; Insulin; Postprandial period; Intervention studies; Phenolic acid; Metagenome; Fatty acids, Volatile


Tämä tutkimus osoitti, että täysjyvä- ja runsaskuituisen leivän vaikutukset verensokeriaineenvaihduntaan välittömyt enemminkin lyhyaikaisten aterianjälkeisten verensokeri- ja insuliinivasteiden kuin tutkittujen paksusuolitapahtumien kautta. Jatkossa ravitsemus- tutkimuksissa pitäisi tarkastella aterianjälkeistä verensokeriaineenvaihduntaa vakioidun ateriakoken avulla paastotilassa tapahtuvien mitausten lisäksi.

Luokitus:
Yleinen Suomalainen asiasanasto: Viljavalmita; Leipä; Ruokana; Verensokeri; Insuliini; Interventio; Fenoliset yhdisteet; Mikrobio; Rasvahapot, paksusuoli
Acknowledgements

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Kuopio, March 2014

Jenni Lappi
List of the original publications

This dissertation is based on the following original publications:


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## Abbreviations

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<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>AACC</td>
<td>American Association of Cereal Chemists</td>
</tr>
<tr>
<td>AACCi</td>
<td>American Association of Cereal Chemists International</td>
</tr>
<tr>
<td>ADA</td>
<td>American Diabetes Association</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the curve</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence interval</td>
</tr>
<tr>
<td>DI</td>
<td>Disposition index</td>
</tr>
<tr>
<td>EFSA</td>
<td>European Food Safety Authority</td>
</tr>
<tr>
<td>FA</td>
<td>Ferulic acid</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>FDR</td>
<td>False discovery rate</td>
</tr>
<tr>
<td>FSGT</td>
<td>Frequently sampled intravenous glucose tolerance test</td>
</tr>
<tr>
<td>GI</td>
<td>Glycaemic index</td>
</tr>
<tr>
<td>GLM</td>
<td>General linear model</td>
</tr>
<tr>
<td>GP</td>
<td>Glycaemic profile</td>
</tr>
<tr>
<td>HDL</td>
<td>High-density lipoprotein cholesterol</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>Homeostatic model assessment of insulin resistance</td>
</tr>
<tr>
<td>HR</td>
<td>Hazard ratio</td>
</tr>
<tr>
<td>IGI</td>
<td>Insulinogenic index</td>
</tr>
<tr>
<td>II</td>
<td>Insulinaemic index</td>
</tr>
<tr>
<td>IFG</td>
<td>Impaired fasting glucose</td>
</tr>
<tr>
<td>IGT</td>
<td>Impaired glucose tolerance</td>
</tr>
<tr>
<td>LDL</td>
<td>Low-density lipoprotein cholesterol</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>NHS</td>
<td>Nurses’ Health Study</td>
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<tr>
<td>OGTT</td>
<td>Oral glucose tolerance test</td>
</tr>
<tr>
<td>OR</td>
<td>Odds ratio</td>
</tr>
<tr>
<td>QUICKI</td>
<td>Quantitative insulin sensitivity check index</td>
</tr>
<tr>
<td>RR</td>
<td>Risk ratio</td>
</tr>
<tr>
<td>SCFA</td>
<td>Short chain fatty acids</td>
</tr>
<tr>
<td>Si</td>
<td>Insulin sensitivity index</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
</tbody>
</table>
1 Introduction

The prevalence of type 2 diabetes among adults is 6% in European countries and 11% in Canada, USA, and Mexico (1). The healthcare costs for diabetes have been estimated to be 10% and 14% of the total healthcare expenditure in Europe and Northern America, respectively, and the costs are estimated to increase 1.2 to 1.4-fold by 2030 (2). The increasing prevalence and healthcare costs of type 2 diabetes call for effective means of prevention of this disease.

Type 2 diabetes is preceded by a prediabetic state when in normal glucose metabolism is disturbed but not so severely as to fulfil the criteria for diabetes. Disturbances in glucose metabolism increase the risk for developing type 2 diabetes (3), but changes in lifestyle, such as improved quality of diet and increased physical activity and reduction in body weight can prevent further deterioration of glucose metabolism to type 2 diabetes (4). Of the single dietary factors associated with the risk of this disease, increased consumption of wholegrain foods and grain fibre has been observed to decrease the risk of type 2 diabetes (5,6). Thus a practical way to decrease the risk of type 2 diabetes may be to simply replace the refined grain foods consumed with the corresponding wholegrain foods.

However, thus far intervention studies of wholegrain foods have failed to conclude that there is a cause-and-effect relationship between the intake of wholegrain foods and improvement in the glucose metabolism. Furthermore, the underlying mechanisms mediating the health effects of wholegrain foods are poorly understood. Improved insulin sensitivity, reduced postprandial blood glucose and insulin responses influenced by food structure, production of short chain fatty acids resulting from fermentation of indigestible carbohydrates, other potential effects on intestinal microbiota, and antioxidative actions of the phenolic compounds present in wholegrains have been presented as potential mechanisms mediating the health effects of wholegrain foods (7).

This doctoral thesis aimed to investigate health effects of wholegrain foods and grain fibre of wheat and rye in healthy subjects and in subjects with the metabolic syndrome, focusing on glucose metabolism and effects in the intestinal tract. In addition to postprandial glucose and insulin responses, effects on intestinal microbiota composition, concentration of short chain fatty acids in peripheral blood, and absorption of phenolic acids and their metabolites from the intestine were investigated.
2 Review of the Literature

2.1 DEFINITION, INTAKE, AND COMPOSITION OF WHOLEGRAIN AND WHOLEGRAIN FOOD

2.1.1 Definitions
The term “wholegrain” means that all the main anatomical parts of the grain are included: bran, endosperm, and germ. Bran is the outer part of the grain and, in turn, consists of three different layers, which are pericarp, testa, and aleurone (Figure 2.1). The outermost layer of the bran, pericarp, contains insoluble fibre and phenolic compounds bound to the cell walls. The second layer, testa, is rich in alkylresorcinols, whereas the innermost layer of the bran, aleurone, contains soluble and insoluble fibre, protein, phenolic compounds, vitamins, and minerals. Endosperm encompasses 80-85% of the weight of the grain and is composed mainly of starch and protein. Germ is the smallest part of the grain containing lipids, vitamins, and minerals.

![Image of wheat grain](image)

*Figure 2.1. Wheat grain (Adapted from Surget and Barron 2005 (8)).*

To help researchers and other specialists, authorities, and consumers to unambiguously identify wholegrains, the American Association of Cereal Chemists (AACC) recommended the following wholegrain definition in 1999: ‘Whole grains shall consist of the intact, ground, cracked or flaked caryopsis, whose principal anatomical components—the starchy endosperm, germ, and bran—are present in the same relative proportions as they exist in the intact caryopsis’ (9). The US Food and Drug Administration (FDA) adopted this definition in 2006 as draft guidance, aiming to assist the industry and manufacturers in labelling grain products (10). The term ‘wholegrains’ includes a variety of grains such as...
wheat, rye, oat, barley, rice, millet, sorghum, and teff, and the so-called pseudo-grains buckwheat, amaranth, and quinoa (11). In 2010, the HealthGrain consortium presented a definition of wholegrains which is similar to the definition of the AACC, but highlights industrial production practices (11). The HealthGrain consortium’s definition allows the removal of the inedible outermost layers of the kernel, such as the hull and the husk. To minimize concentrations of undesirable substances such as bacteria, moulds, and heavy metals, small losses of grain components that occur through processing methods are allowed. Thus, less than 2% of the grain or 10% of the bran can be removed in processing. Furthermore, this definition accepts the separation of endosperm, bran, and germ temporarily during processing for later recombination, provided that the constituents in the end product are in the same proportions as in the original grain. It should be noted that the guidance of the AACC, FDA, and HealthGrain consortium for the definition of wholegrain are recommendations and are not legally binding.

The definition of wholegrains is unambiguous, but what is considered a wholegrain food is not clear-cut and varies across countries. Furthermore, only a few countries have an official definition for wholegrain foods. US dietary guidelines provide information on how to identify foods that contain a substantial amount of wholegrains (12). Thus, when a food contains at least 51% of wholegrain ingredients by fresh weight per the reference amount customarily consumed, it is a good source of wholegrains. The amount customarily consumed is, for example, 1 slice (30 g) of bread or approximately 30 g (1 ounce) of ready-to-eat breakfast cereals. Recently, AACC International (AACC) released a definition of a wholegrain food (13): such a food product must contain at least 8 g of wholegrain per 30 g of a fresh product. To help consumers to identify wholegrain products with substantial amounts of wholegrains, a wholegrain stamp was developed in the US and adopted by several countries (14). However, use of the stamp is voluntary, and not all wholegrain products are labelled with it.

In Denmark, the wholegrain campaign has promoted the use of the national wholegrain logo in product labelling. The specifications of the wholegrain products permitted to use the wholegrain logo differ from those in the US. For example, the logo can be used in labelling bread if the bread contains at least 50% of wholegrains based on dry matter, or 30% of total weight (15). In the Netherlands, the law states that breads can be labelled as wholegrain if 100% of the grain ingredients used is wholegrain (16). A summary of the existing definitions of wholegrain products in different countries is presented by the United States' Whole Grains Council (16). All in all, the definition of wholegrain products varies significantly among countries. In Finland, Leipätiedotus ry (Finnish Bread Information) suggests that wholegrain bread should contain at least 50% wholegrains of the total grain ingredients used. However, the practice in labelling bread varies among the manufacturers, and bread may be called wholegrain even with lower amounts of wholegrains. Despite the name of a product, consumers should always read the list of ingredients in order to assess whether a product is a good source of wholegrains.

2.1.2 Recommended and actual wholegrain intake
Dietary recommendations for wholegrain intake are generally based on evidence from prospective cohort studies on the association of the intake of wholegrain foods with the risk
of type 2 diabetes (17) and cardiovascular diseases (18). These studies, with a total of 433,861 subjects, demonstrate that those who consume wholegrain foods have 21-31% lower risk of developing type 2 diabetes as compared to those who do not consume wholegrain foods (17,19-24) (Table 2.1). The decreased risk of type 2 diabetes is observed when approximately three servings of wholegrain foods are consumed daily (19-21,23).

The recently updated Finnish nutrition recommendations include eating six to nine portions of grain foods daily so that at least half of the consumed portions is of wholegrains (25). However, wholegrain foods are not specified as in the US dietary guidelines (12), except for wholegrain bread which is defined as a bread containing at least 50% of wholegrains of the total grain ingredients (25). The US guidelines recommend the use of at least three to five portions of wholegrain foods daily, while the Danish and Swedish nutrition recommendations emphasize the consumption of a more fixed amount of wholegrains daily, 75 g and 70-90 g, respectively, as wholegrain ingredients from different food sources (26,27). All in all, there is no commonly agreed international recommendation for the consumption of wholegrain foods, and recommendations vary from country to country.

**Wholegrain intake in epidemiological studies**

The US dietary guidelines, which recommend consuming at least three portions of wholegrain foods daily, are based on the evidence from seven prospective cohort studies, of which six were conducted in the USA (Table 2.1). In general, the reported consumption of the wholegrain foods in the North American studies is low as compared to the Finnish study. In one American study (24), the daily intake of wholegrain foods in the highest quartile was only 1.3 servings and the risk of type 2 diabetes was less as compared to individuals who did not consume wholegrain foods. One of the studies conducted in the USA reports the wholegrain intake as ingredients and shows that the risk of type 2 diabetes decreased with the consumption of 31 g/d of wholegrain ingredients as compared to the consumption of hardly any wholegrain ingredients (17). If assumed that one serving (30 g) of a wholegrain food contains at least 51% of wholegrain ingredients, the 31 g of wholegrain ingredients makes up two daily servings of wholegrain foods. In a Finnish study by Montonen et al. (22), the daily consumption of wholegrain foods was as high as 302 g (i.e. 10 servings) in the highest quartile of intake. When the highest quartile was compared with the lowest, there was actually no statistically significant association with the risk of type 2 diabetes (risk ratio 0.65, 95% confidence interval (CI) 0.36-1.18) although the trend across the quartiles was statistically significant (p=0.02). However, when the third quartile (198 g/d) was compared with the lowest quartile (79 g/d), the risk of type 2 diabetes was significantly decreased (risk ratio 0.52, 95% CI 0.31-0.88). A Swedish study reported higher intake of wholegrains than the studies conducted in the USA: a decreased risk (22%; p=0.024) of developing type 2 diabetes or prediabetes (that is impaired fasting glucose (IFG) or impaired glucose tolerance (IGT), or both) was observed when more than 59 g as compared to less than 31 g of wholegrain ingredients was consumed daily (28).

The association of wholegrain intake with the risk of type 2 diabetes has been adjusted for several confounding factors, i.e. age, body mass index (BMI), physical activity, smoking, family history of diabetes, total energy intake, and alcohol consumption in the statistical
models of most of the epidemiological studies. Montonen et al. (22) did not control the results for physical activity and alcohol consumption, and both Montonen et al. (22) and Meyer et al (21) did not control the results for family history of diabetes. However, in all these studies individuals with the highest consumption of wholegrain foods tended to be leaner, physically more active and non-smokers. Thus, consumption of wholegrain foods seems to be a good marker for healthy lifestyle.

Recommending specific wholegrain foods based on prospective cohort studies is not justified because cohort studies define vaguely foods that can be categorized as wholegrain foods (Table 2.1). Five studies defined dark bread as a wholegrain food (19-21,23,24) although no information was provided whether dark breads included wholegrain ingredients or bran or, for example, molasses to colour the bread. Several studies included bran in the category of wholegrain foods (19-21,24), and three studies regarded breakfast cereals including at least 25% of wholegrain ingredients or bran as wholegrain foods (19-21). Popcorn was also regarded as wholegrain (17,19-21). Thus, 75% of the ingredients in the cereals studied might have been from refined grains.

Categories to define wholegrain foods similar to those described above have been used in cross-sectional studies investigating the association of wholegrain consumption with glucose metabolism (29-32). A Swedish prospective cohort study (28) included only grain foods that contained ≥ 50% of wholegrain ingredients per serving, which might have caused underestimation of the total wholegrain intake. De Munter et al. (17), Jensen et al. (33) and Newby et al. (34) reported the intake of wholegrains as ingredients, which is an accurate method to calculate intake of wholegrains from all grain and/or grain containing foods. In this case, there is no need to specify the individual wholegrain foods according to their wholegrain content.

The wholegrain consumed in the prospective cohort studies has been mainly wheat as most of them have been conducted in the USA. In addition to wheat, oat has been consumed as a cold or cooked breakfast cereal in the USA. However, in the Finnish study (22), the most consumed wholegrain was rye.

Health claims for wholegrain foods
European Food Safety Authority (EFSA) has rejected health claims for wholegrain foods with a statement that wholegrain foods are not sufficiently characterized and a-cause-and-effect relationship is inadequate to support a health claim (35). However, in the USA, FDA has approved a wholegrain health claim in 1999 to be used in product labelling stating ‘Diets rich in whole grain foods and other plant foods and low in total fat, saturated fat, and cholesterol, may help reduce the risk of heart disease and certain cancers’ (36). In order to use this health claim, the food or product must fulfil the following criterion: it has to contain at least 51% of wholegrain ingredients by fresh weight per reference amount customarily consumed. The health claim used in the USA is based on the evidence from prospective cohort studies.
National intake of wholegrains

Despite the dietary recommendations and evidence from the prospective cohort studies, adults in the USA consume on average a half serving (0.5 ounce equivalents) of wholegrain foods daily, which is one sixth of the recommended intake (37). At the turn of the century, 72% of US adults consumed daily 0-0.6 servings of wholegrain foods (38). At the same time, 29% of the British did not consume wholegrains at all, when the national average (median) amount of wholegrain ingredients consumed was 14 g/d (approximately one serving) (39). Adults in Sweden, Norway, and Denmark, on the other hand, have been reported to consume 31-44 g/41-49 g (women/men) wholegrains daily (as wholegrain ingredients) (40). There is no data on consumption of wholegrains in Finland. These data indicate that the consumption of wholegrain foods is very low in the USA and UK, while in Scandinavia the intake is close to that recommended in the USA.

Wholegrain foods versus grain fibre

Recommendating specific wholegrain foods is not justified based on results from prospective cohort studies, but it is also questionable whether the recommendations should underline intake of wholegrain foods or actually grain fibre. When the association of wholegrain intake with risk of type 2 diabetes observed in the prospective cohort studies was adjusted for cereal (i.e. grain) fibre intake in the statistical models, the association was no more significant (19,21,22). Thus, the association between wholegrain food intake and type 2 diabetes is explained by grain fibre intake. Furthermore, de Munter et al. (17) observed that the association between wholegrain intake and type 2 diabetes was similar to that between bran intake and type 2 diabetes. That bran is high in grain fibre might explain the effect.

Prospective cohort studies investigating the association of grain fibre intake with the risk of type 2 diabetes show decreased risk with increased intake of grain fibre (Table 2.2). The only such study not showing such a correlation is an Australian study in which the authors admit they were not able to calculate exact grain fibre intake because the information regarding content of fibre in breakfast cereals was unavailable (41). All in all, it seems that grain fibre is an important mediator of the effect of wholegrain foods on type 2 diabetes.
<table>
<thead>
<tr>
<th>Year of study, country (reference)</th>
<th>Population</th>
<th>Wholegrain foods</th>
<th>Intake of wholegrain foods/d(^4)</th>
<th>Results (RR/HR(CI))(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2000, USA (20) 75 521 women</td>
<td>Wholegrain breakfast cereal, dark bread, popcorn, cooked oatmeal, wheat germ, brown rice, bran, and other grains (e.g. bulgur, kasha, couscous). Breakfast cereals were regarded as wholegrain if the product contained ≥ 25% wholegrains or bran by weight.</td>
<td>0.1 vs. 2.7 (servings)</td>
<td>0.73 (0.63:0.85) p for trend &lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>2000, USA (21) 35 988 women</td>
<td>Wholegrain breakfast cereal, dark bread, popcorn, cooked oatmeal, wheat germ, brown rice, bran, and other grains (e.g. bulgur, kasha, couscous). Breakfast cereals were regarded as wholegrain if the product contained ≥ 25% wholegrains or bran by weight.</td>
<td>0.1 vs. 2.9 (servings)</td>
<td>0.79 (0.65:0.96) p for trend 0.0089</td>
<td></td>
</tr>
<tr>
<td>2002, USA (19) 42 898 men</td>
<td>Wholegrain breakfast cereal, dark bread, popcorn, cooked oatmeal, wheat germ, brown rice, bran, and other grains (e.g. bulgur, kasha, couscous). Breakfast cereals were regarded as wholegrain if the product contained ≥ 25% wholegrains or bran by weight.</td>
<td>0.4 vs. 3.2 (servings)</td>
<td>0.70 (0.57:0.85) p for trend 0.0006</td>
<td></td>
</tr>
<tr>
<td>2003, Finland (22) 4316 men and women</td>
<td>Only grains included; rye bread, all wholegrain flours and other products (rye, whole wheat, wheat germ, rolled oats, barley, millet, buckwheat) derived from different grain foods, breads prepared from mixtures of wholegrains and refined grains (proportion of wholegrain flour 25-50%)</td>
<td>79 vs. 302 (g)</td>
<td>0.65 (0.36:1.18) p for trend 0.02</td>
<td></td>
</tr>
<tr>
<td>2006, USA (24) 41 186 women</td>
<td>High fibre, bran, granola, and shredded wheat breakfast cereals, dark bread such as wheat, rye, and pumpernickel bread</td>
<td>0.03 vs. 1.29 (servings)</td>
<td>0.69 (0.60:0.79) p for trend &lt; 0.0001</td>
<td></td>
</tr>
<tr>
<td>2007, USA (17) 161 737 women</td>
<td>Whole wheat and whole wheat flour, whole oats and whole oat flour, whole cornmeal and whole corn flour, brown rice and brown rice flour, whole rye and whole rye flour, whole barley, bulgur, buckwheat, popcorn, amaranth, psyllium</td>
<td>NHS(^3) I: 3.7 vs. 31.2 (3.7 vs. 31.2) p for trend &lt;0.001</td>
<td>0.75 (0.68:0.83) p for trend &lt;0.001</td>
<td></td>
</tr>
<tr>
<td>2013, USA (23) 72 215 women</td>
<td>dark bread such as wheat, rye, and pumpernickel bread, cold and cooked wholegrain breakfast cereal (not specified whether high-fibre and bran cereals were included), not specified whether popcorn was included</td>
<td>0.18 vs. 2.59 (servings)</td>
<td>0.75 (0.63:0.89) p for trend 0.0139</td>
<td></td>
</tr>
</tbody>
</table>

\(^1\)median of the lowest vs. the highest quintile/quartile; \(^2\)RR, risk ratio; HR, hazard ratio; CI, 95% confidence interval; \(^3\)NHS, Nurses’ Health Study
Table 2.2. Consumption of grain fibre associated with risk of type 2 diabetes

<table>
<thead>
<tr>
<th>Year of study, country (reference)</th>
<th>Population</th>
<th>Intake of grain fibre (g/d)¹</th>
<th>Results (RR/OR/HR (CI))²</th>
</tr>
</thead>
<tbody>
<tr>
<td>1997, USA (42)</td>
<td>42,759 men</td>
<td>2.5 vs. 10.2</td>
<td>0.70 (0.51;0.96) p for trend 0.007</td>
</tr>
<tr>
<td>1997, USA (43)</td>
<td>65,173 women</td>
<td>2.0 vs. 7.5</td>
<td>0.72 (0.58;0.9) p for trend 0.001</td>
</tr>
<tr>
<td>2000, USA (21)</td>
<td>35,988 women</td>
<td>2.7 vs. 9.4</td>
<td>0.64 (0.53;0.79) p for trend 0.0001</td>
</tr>
<tr>
<td>2001, USA (44)</td>
<td>84,941 women</td>
<td>not reported</td>
<td>0.6 (~ 0.5;0.7) p for trend &lt; 0.001</td>
</tr>
<tr>
<td>2002, USA (45)</td>
<td>12,251 men and women (9,529 whites and 2,722 African-Americans)</td>
<td>not reported</td>
<td>in whites: 0.75 (0.60;0.92) in African-Americans: 0.86 (0.65;1.15)</td>
</tr>
<tr>
<td>2003, Finland (22)</td>
<td>4,316 men and women</td>
<td>0.47-12.0 vs. 24.5-111.0 g (ranges)</td>
<td>0.39 (0.20;0.77) p for trend 0.01</td>
</tr>
<tr>
<td>2004, USA (46)</td>
<td>91,249 women</td>
<td>3.1 vs. 8.8</td>
<td>0.64 (0.48;0.86) p for trend 0.004</td>
</tr>
<tr>
<td>2004, Australia (41)</td>
<td>31,641 men and women</td>
<td>0 vs. 10</td>
<td>1.08 (0.88;1.32) p for trend 0.46</td>
</tr>
<tr>
<td>2007, Germany (5)</td>
<td>25,067 men and women</td>
<td>6.6 vs. 16.6</td>
<td>0.72 (0.56;0.93) p for trend 0.02</td>
</tr>
</tbody>
</table>

¹median of the lowest vs. the highest quintile/quartile; ²RR, risk ratio; OR, odds ratio; HR, hazard ratio; CI, 95% confidence interval
2.1.3 Structure and components of cereal foods
From the beginning of the agricultural revolution 10 000 years ago, using grains as food has been common among human beings. Before the industrial revolution in the 19th century all parts of the grain were used for food. However, during the industrial revolution, milling of grains developed due to the introduction of high-speed roller mills. Grains were effectively ground to fine flour and the bran was removed to increase palatability and yield. Unfortunately the desire to increase palatability simultaneously decreased the nutritional quality of the grains. When the bran and germ are removed in the refining process, the contents of fibre and nutrients are markedly reduced as compared to the corresponding non-refined wholegrain (47). Furthermore, increased digestibility of starch after ingestion of grain products made of finely ground flour as compared to those made mostly of whole kernels increases postprandial glucose and insulin responses, probably increasing the risk of disturbances of glucose metabolism (48).

Grains need to be processed to make products suitable for human consumption. Processing alters the structure and composition of the original grain. At the least, the hull or husk is removed, but the usual processing of grains covers several practices the most common of which is milling which was initially done manually and utilized by the ancient Mesopotamians to bake the first leavened breads (49). Pretreatments prior to cooking or baking may also include germinating and fermentation or other sophisticated cereal technology processing to improve the nutritional quality, safety and sensory properties of the final product (50). For example, sourdough fermentation of wholegrain flour increases the content of water-soluble phenolic compounds and folate and degrades proteins and fructan (51,52).

Grain fibre denotes all fibre located in the bran and endosperm. Wholegrain wheat and rye contain, on average, 14 and 18% total fibre (without fructan), respectively (53). The main grain fibre components are arabinoxylan, beta-glucan, fructan, and cellulose (54-56). As compared to wheat, rye contains more total arabinoxylan (3.6 vs. 1.9%), soluble arabinoxylan (i.e. water-extractable arabinoxylan) (1.2 vs. 0.4%), beta-glucan (1.8 vs. 0.5%), and fructan (4.6-6.6 vs. 1.3%) (percentages on dry weight basis) (54,55,57,58). Thus, rye contains more soluble fibre (sum of soluble arabinoxylan, beta-glucan, and fructan) than wheat. Soluble arabinoxylan, and especially the high molecular weight arabinoxylan, increases the viscosity of rye flour when mixed with water (59). There is a higher proportion of soluble arabinoxylan in rye endosperm than in other parts of the grain (60). Furthermore, rye fibre is more evenly distributed in the grain (60), which is why refined endosperm rye bread contains more fibre (on average 7%) than refined endosperm wheat bread (on average 3%) (61,62). Endosperm rye bread is an uncommon example of a product which is high in grain fibre although it does not contain bran.

Most of the grain fibre is concentrated in the bran (53). Wheat and rye brans contain, on average, 44-47 and 36-44% of fibre, respectively (53,63). The total arabinoxylan content is similar in rye and wheat bran, 13-25% depending on the method used to analyse arabinoxylan (54,55,63). The proportion of the soluble arabinoxylan is higher in rye bran (1.3%) than in wheat bran (0.4%) (54,55). Rye bran contains 4.7% of beta-glucan, 6.8% of
fructan, and 5.8% of cellulose, whereas wheat bran contains 2.4% of beta-glucan, 3.3% of fructan, and 10.7% of cellulose (63).

According to the definitions of dietary fibre issued by the European Union and Codex Alimentarius Commission (Codex), phenolic compounds naturally associated with carbohydrate polymers of plant origin are part of fibre (64,65). Phenolic acids are a group of phenolic compounds concentrated in the bran fraction of the wheat and rye grain (63,66). Of the phenolic acids, the most abundant ones in quantity in wheat and rye are ferulic acid (FA) followed by sinapic and p-coumaric acids (55,66,67). Phenolic compounds include also alkylresorcinols which are found in high concentrations in wheat and rye bran (55,68), and are considered to be a good biomarker of wholegrain wheat and rye intake (69).

### 2.2 LINKING WHOLEGRAIN FOODS AND GRAIN FIBRE WITH DISTURBANCES IN GLUCOSE METABOLISM

#### 2.2.1 Disturbances in glucose metabolism

When normal glucose metabolism is disturbed, either fasting or postprandial concentration of blood glucose increases. Increased concentration of fasting glucose is called ‘impaired fasting glucose’ (IFG), and increased concentration of 2-hour glucose concentration in oral glucose tolerance test (OGTT) is called ‘impaired glucose tolerance’ (IGT). Also, both fasting and 2-hour postprandial glucose concentrations can be elevated in a more severe abnormality of glucose metabolism in the case of combined IFG and IGT. The American Diabetes Association (ADA) (70), the World Health Organization (WHO) (71), and the Finnish Current Care Guidelines (i.e. Käypiä hoito -suositukset in Finnish) (72) have set the criteria for disturbed glucose metabolism, the only difference between the criteria being the lower limit in fasting plasma glucose concentration of IFG, which is 5.6 mmol/l according to the ADA and 6.1 mmol/l according to the WHO and the Finnish Current Care Guidelines (Table 2.3).

<table>
<thead>
<tr>
<th>Category</th>
<th>Fasting plasma glucose</th>
<th>2-hour plasma glucose&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>&lt; 5.6 mmol/l</td>
<td>&lt; 7.8 mmol/l</td>
</tr>
<tr>
<td></td>
<td>&lt; 6.1 mmol/l&lt;sup&gt;2,3&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>IFG</td>
<td>5.6 – 6.9 mmol/l</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>6.1 – 6.9 mmol/l&lt;sup&gt;2,3&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>IGT</td>
<td>-</td>
<td>7.8 – 11 mmol/l</td>
</tr>
<tr>
<td>Diabetes</td>
<td>≥ 7.0 mmol/l</td>
<td>≥ 11.1 mmol/l</td>
</tr>
</tbody>
</table>

<sup>1</sup> Oral glucose tolerance test (OGTT);<sup>2</sup> Criteria according to the World Health Organization (71);<sup>3</sup> Criteria according to the Finnish Current Care Guidelines for diabetes (72); IFG, impaired fasting glucose; IGT, impaired glucose tolerance
IFG and IGT seem to differ in pathophysiology and aetiology (73,74). Peripheral, i.e. muscle, insulin sensitivity is decreased in individuals with IGT, but not in those with IFG. Thus, when peripheral insulin sensitivity is decreased, uptake of glucose from circulation to muscle is less efficient and postprandial glucose levels rise. In IFG, instead, decreased hepatic insulin sensitivity plays a major role by impairing suppression of hepatic glucose production. IFG and IGT are also characterized by pancreatic beta cell dysfunction, which leads to decreased capacity to acutely secrete insulin (i.e. decreased first-phase insulin secretion) (73).

Lack of physical activity and poor quality of diet decrease peripheral insulin sensitivity (74). This is why physical inactivity and adverse dietary habits may lead to the development of IGT. Smoking and genetic factors seem to be major contributors to the development of IFG. IFG and IGT are intermediate phases between normal glucose tolerance and diabetes, and are often called prediabetic states. Both increase the risk for developing type 2 diabetes (3).

IFG is also a component of the metabolic syndrome, which is a cluster of metabolic abnormalities including elevated fasting glucose concentration (or drug treatment for elevated glucose), elevated waist circumference (illustrating abdominal obesity), elevated concentration of triglycerides (or drug treatment for elevated triglycerides), reduced concentration of high-density lipoprotein cholesterol (HDL) (or drug treatment for reduced HDL), and hypertension (or drug treatment for elevated blood pressure) (75). To label a condition as the metabolic syndrome, several major organizations (such as the International Diabetes Federation, the American Heart Association, and the National Heart, Lung, and Blood Institute) have agreed that three out of the five criteria described in Table 2.4 should be met. The major organizations agree that the metabolic syndrome increases the risk for type 2 diabetes and cardiovascular diseases.

Table 2.4. Criteria for the metabolic syndrome. Three out of five criteria should be fulfilled (75).

<table>
<thead>
<tr>
<th>Measure</th>
<th>Cut points</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elevated concentration of fasting glucose (IFG)</td>
<td>≥ 5.6 mmol/l</td>
</tr>
<tr>
<td>Elevated concentration of triglycerides</td>
<td>≥ 1.7 mmol/l</td>
</tr>
<tr>
<td>Reduced concentration of HDL</td>
<td>&lt; 1.0 mmol/l (men); &lt; 1.3 mmol/l (women)</td>
</tr>
<tr>
<td>Elevated blood pressure</td>
<td>≥ 130/85 mmHg</td>
</tr>
<tr>
<td>Elevated waist circumference</td>
<td>Population- or country-specific cut points¹</td>
</tr>
</tbody>
</table>

¹ Asian populations ≥ 90 cm / ≥ 80 cm (men/women), Japanese ≥ 85 cm / ≥ 90 cm (men/women)); individuals of European origin either ≥ 94 cm / ≥ 80 cm (men/women) or ≥ 102 cm / ≥ 88 cm (men/women) (76,77)

Type 2 diabetes is characterized by severe impairment in insulin action and insulin secretion which results in chronic hyperglycemia (3). It has been demonstrated that changes in lifestyle, such as increasing quality of diet and physical activity, decrease the risk of type 2 diabetes in individuals with IGT (4).
Measurement of glucose metabolism

Several techniques can be used to measure insulin sensitivity and insulin secretion. The hyperinsulinemic-euglycemic clamp is considered the gold standard (78). In this test, insulin and glucose are given intravenously, and hepatic endogenous glucose production is suppressed by the high insulin bolus. Consequently, 80-90% of glucose is taken up by the muscles, which is why the technique mainly reflects peripheral insulin sensitivity (73). The frequently sampled intravenous glucose tolerance test (FSIGT) measures insulin sensitivity indirectly (78) and reflects both hepatic and peripheral insulin sensitivity (73).

Insulin sensitivity can also be measured in OGTT by administering glucose orally, or using the fasting values of blood glucose and insulin. Indexes calculated from glucose and insulin concentrations during OGTT reflect both hepatic and peripheral insulin sensitivity, i.e. whole-body insulin sensitivity. In fasting state, when endogenous glucose production mainly determines blood concentrations of glucose and insulin, hepatic insulin sensitivity can be evaluated by calculating the indexes of homeostatic model assessment of insulin resistance (HOMA-IR) and quantitative insulin sensitivity check index (QUICKI) (73).

Insulin secretion capacity can be measured from both intravenous tests and OGTT. In both FSIGT and the hyperglycemic clamp techniques the rapid increase in plasma glucose concentration rapidly and transiently affects insulin secretion (73). Thus, first-phase insulin secretion or ‘acute insulin response’ can be calculated from the insulin concentrations during the first 10 min. First-phase insulin secretion can be evaluated also from OGTT by calculating the increment in blood insulin relative to the increment in blood glucose using the fasting and 30 min glucose and insulin concentrations. Insulin secretion capacity in some studies has been related to the state of peripheral insulin sensitivity of individuals (73). Thus, when disposition index (DI) is calculated, it describes first-phase or acute insulin secretion capacity in relation to insulin sensitivity.

2.2.2 Findings from epidemiological studies

Cross-sectional and prospective epidemiological studies have investigated the association of wholegrain foods with some markers of glucose metabolism such as insulin sensitivity, fasting glucose and insulin concentrations, and 2-hour glucose concentration measured in OGTT. Improved insulin sensitivity was observed to be associated with higher consumption of wholegrain foods in adults when measured by FSIGT (29) or homeostatic model assessment (HOMA) (28,30), and in adolescents when measured by the euglycemic insulin clamp method (32). Adjustment for BMI attenuated the effect, but the association was still significant (28,29,32).

A meta-analysis including approximately 48 000 European individuals showed a strong inverse relationship between the intake of wholegrain foods and fasting glucose and insulin concentrations, even when adjusted for BMI (79). However, the reported lower concentration of fasting glucose (-0.009 mmol/l) and insulin (-0.011 pmol/l) associated with a higher intake of one serving of wholegrain foods seems clinically insignificant. Other cross-sectional studies show no association between wholegrain food intake and fasting glucose concentration (30-32,34) or fasting insulin concentration (29,32-34) after appropriate
adjustment for BMI although one study observed that higher intake of wholegrain foods was associated with lower fasting insulin concentration even when adjusted for BMI (31). The same study (31) showed no association between the intake of wholegrain foods and 2-hour glucose concentration, but another study observed an inverse association even when the analysis was adjusted for BMI (34). Recently it was reported in Swedish adults that the risk of progress from normal glucose tolerance to IFG or IGT over eight to ten years of follow-up was decreased by 12% (p=0.015) per 30 g daily intake of wholegrain ingredients (28). The decreased risk was observed in men but not in women.

2.2.3 Findings from intervention and postprandial studies

Intervention studies

Intervention studies can generally only be conducted to investigate the risk markers of a chronic disease, because appearance of the disease may take several years or decades. Epidemiological studies investigating the role of wholegrains in the risk of type 2 diabetes or in glucose metabolism suggest an effect on glycaemic response (19-21,29,31,34) and/or on insulin sensitivity (19,24,31,33) which could explain the beneficial effects of wholegrain foods. However, wholegrain foods made of milled flour do not produce a low glycaemic response (see the following section Postprandial studies). A ten-week intervention study was conducted to investigate the role of mere glycaemic response of wholegrain foods when intake of grain fibre as a confounding factor was excluded (80). The glycaemic response of wholegrain foods was manipulated by baking wheat and rye breads with intact whole kernels in the low-glycaemic index (GI) wholegrain diet and with wholemeal flour in the high-GI wholegrain diet. Insulin sensitivity calculated by HOMA was the same between the diet groups. This one study suggests that the glycaemic response of wholegrain foods is not the main factor influencing glucose metabolism, but additional studies are needed to confirm the suggestion.

Insulin sensitivity has been investigated in a few intervention studies in which mainly whole wheat and/or rye products have been consumed as compared to refined grain foods. Insulin sensitivity was measured in nine studies of which only two observed a beneficial effect due to consumption of wholegrains (Table 2.5). Pereira et al. (81) used the euglycemic hyperinsulinemic clamp technique and observed improved insulin sensitivity in overweight subjects with hyperinsulinemia consuming a diet containing wholegrain foods as compared with refined grain foods. Rave et al. (82) evaluated insulin sensitivity using the HOMA-IR and found improved insulin sensitivity in obese subjects with IFG consuming a wholegrain-based dietary product as a meal replacement. However, five studies show that consuming wholegrain foods does not affect insulin sensitivity when insulin sensitivity is evaluated from fasting glucose and insulin concentrations of overweight but healthy subjects or subjects with metabolic syndrome or features of metabolic syndrome (83-87). One study did not report insulin sensitivity but found lower fasting insulin concentration after wholegrain rye and rye bran products were consumed, compared to the control diet (88). However, the intake of rye fibre in that study with elderly subjects having a history of prostate cancer was exceptionally high (58 g/d) which makes comparison with other studies difficult. Andersson et al. (89) and Juntunen et al. (90) used intravenous tests but did not observe improvement in insulin sensitivity in overweight but
healthy subjects after these subjects consumed wholegrain foods and foods rich in grain fibre. These results suggest that insulin sensitivity measured at fasting state is not affected by consumption of conventional wholegrain foods. Furthermore, improvement in insulin sensitivity measured intravenously may be observed only in overweight subjects with early disturbances in glucose metabolism such as hyperinsulinemia.

Acute insulin response or first-phase insulin secretion has been measured in two Finnish studies and in one Canadian study in which the control diet was based on refined grains (86,90,91). The Finnish study in healthy postmenopausal women showed improved acute insulin response measured in FSIGT after subjects' consumption of a diet containing wholegrain rye bread enriched with rye bran as compared to the control diet with refined wheat bread (90). Another Finnish study found improved first-phase insulin secretion measured in OGTT in subjects with IFG or/and IGT and metabolic syndrome (92). In that study, the control diet was not based on refined grains but on a mixture of breads baked with refined and whole wheat and oat and wheat bran inducing a higher postprandial insulin response than the breads in the test diet. In a third Finnish study, OGTT was used, and the subjects had IFG or IGT and features of the metabolic syndrome (86). No improvement in first-phase insulin secretion was observed after subjects' consumption of a diet based on wholegrain and rye-fibre-rich foods, although the test diet was similar to that in the study by Laaksonen et al. (92). Furthermore, the Canadian study, using OGTT, showed no effects on fasting values but a trend for improved glucose response and first-phase insulin secretion in subjects with IFG or IGT after consumption of sourdough wholegrain wheat bread as compared to that of refined white wheat bread (91). Characteristics of the subjects with IFG or IGT in all of these three studies were similar, except the absence of cholesterol-lowering medication in the studies by Laaksonen et al. (92) and Mackay et al. (91), and a slightly higher total and LDL cholesterol in the study by Laaksonen et al. (92) as compared to that by Lankinen et al. (86) in which 28% of the subjects used statins.

To the best of our knowledge, there are no studies investigating the effects of wheat or rye bran-based diets as compared to an appropriate low grain-fibre control diet on glucose metabolism in healthy subjects or in subjects with IFG, IGT, or metabolic syndrome. Instead, a crossover study in 23 diabetic subjects treated with diet or with a combination of diet and oral hypoglycemic drugs showed no difference in fasting glucose concentration as a result of a three-month consumption of wheat bran bread and breakfast cereals providing 19 g of fibre as compared to that of white wheat bread and low-fibre breakfast cereal providing 4 g of fibre (93).
<table>
<thead>
<tr>
<th>Year of study, country (reference)</th>
<th>Subjects (n, age, BMI, health status)</th>
<th>Study design</th>
<th>Wholegrain products (intake/d)</th>
<th>Refined grain products (intake/d)</th>
<th>Intake of wholegrain ingredients/d</th>
<th>Results(^1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2000, Finland (94)</td>
<td>22 women, 18 men men, 43 ± 2.0 y, 25.7 ± 0.8 kg/m(^2); women, 43 ± 1.6 y, 23.6 ± 0.5 kg/m(^2) (mean ± SEM) elevated total cholesterol</td>
<td>randomized crossover; 2 x 4 wk</td>
<td>rye bread: 6.7 ± 0.4 portions (219 ± 14.6 g) by men; 4.9 ± 0.2 portions (163 ± 5.1 g) by women (mean ± SEM)</td>
<td>white wheat bread: 8.3 ± 0.4 portions (200 ± 9.6 g) by men; 6.4 ± 0.2 portions (152 ± 5.6 g) by women (mean ± SEM)</td>
<td>no data</td>
<td>no difference in fasting glucose and insulin</td>
</tr>
<tr>
<td>2002, USA (81)</td>
<td>6 women, 5 men; 25-56 y; 30.2 ± 1.0 kg/m(^2) (mean ± SEM) hyperinsulinemia</td>
<td>randomized crossover; 2 x 6 wk</td>
<td>altogether 6-10 servings: 1 serving is e.g. 30 g of breakfast cereal, 30 g of bread, 140 g of cooked pasta, 75 g of muffin, 25 g of cookies, 26 g of snacks source of grain was 80% wheat; the remainder was oats, rice, corn, barley, rye; milled to flour</td>
<td>wheat, rice, and corn with no bran and germ, little fibre</td>
<td>no data</td>
<td>fasting insulin concentration decreased and insulin sensitivity improved(^7)</td>
</tr>
<tr>
<td>2003, Finland (90)</td>
<td>20 women; 59 ± 6 y; 27.5 ± 2.9 kg/m(^2) (mean ± SD) healthy with elevated total cholesterol (3 IGT)</td>
<td>randomized crossover; 2 x 8 wk</td>
<td>rye bread enriched with rye bran, minimum of 4-5 portions (≥ 96-140 g)</td>
<td>white wheat bread 83-125 g</td>
<td>no data</td>
<td>acute insulin response increased, no effect on insulin sensitivity(^8)</td>
</tr>
<tr>
<td>2003, Australia (95)</td>
<td>28 men; 40-65 y; 30 ± 0.9 kg/m(^2) (mean ± SEM) No information about glucose metabolism</td>
<td>randomized crossover; 3 x 4 wk</td>
<td>rye and wheat wholegrain diets with 135 g wholemeal bread, 22 g rye crisp bread/42 g wheat crisp bread, 50 g breakfast cereal</td>
<td>135 g white wheat bread, 42 g refined wheat crispbread, 50 g rice cereal; low fibre content</td>
<td>88 g</td>
<td>no difference in fasting glucose and insulin concentrations</td>
</tr>
</tbody>
</table>

(Table continues on the next page)
| Year of study, country (reference) | Subjects (n, y, BMI, health status) | Study design | Wholegrain products (intake/d) | Refined grain products (intake/d) | Intake of wholegrain ingredients/d | Results  
<table>
<thead>
<tr>
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</thead>
<tbody>
<tr>
<td>2007, Sweden (89)</td>
<td>22 women, 8 men; 59 ± 5 y; 28.3 ± 2.0 kg/m² (mean ± SD)</td>
<td>randomized crossover; 2 x 6 wk</td>
<td>wheat, rye and oat products with ≥ 50% wholegrain per dry weight, mainly in milled form:</td>
<td>refined wheat, rye, and com products</td>
<td>101-112 g</td>
<td>no difference in insulin sensitivity³ and fasting glucose and insulin concentrations</td>
</tr>
<tr>
<td></td>
<td>healthy</td>
<td></td>
<td>135 g of bread, 24 g of crisp bread, 35 g of muesli, 70 g of pasta</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>2007, Germany (82)⁵</td>
<td>13 men, 18 women; 51 ± 13 y, 33.9 ± 2.7 kg/m² (mean ± SD)</td>
<td>randomized crossover; 2 x 4 wk</td>
<td>188 ± 44 g (mean ± SD) of wholegrain-based dietary product with reduced starch content (Balantose™), (contained fibre naturally occurring in wholegrain wheat)</td>
<td>188 ± 44 g (mean ± SD) of a product rich in carbohydrates (Slim Fast™), (contained a mixture of added fibre, mainly inulin)</td>
<td>no data</td>
<td>insulin sensitivity⁶ increased and fasting insulin decreased more from baseline</td>
</tr>
<tr>
<td></td>
<td>IFG</td>
<td></td>
<td>(2 daily meals were replaced with the product)</td>
<td>(2 daily meals were replaced with the product)</td>
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<tr>
<td>2008, USA (85)⁵</td>
<td>23 women, 24 men; 45±8 y (WG group), 47 ± 10 y (refined grain group); 36 ± 4 kg/m² (WG group), 36 ± 5 kg/m² (refined grain group) (mean ± SD)</td>
<td>randomized parallel; 12 wk</td>
<td>primary sources bread and rolls other sources ready-to-eat cereal, brown rice, oatmeal, pasta, salty snacks and snack bars; products with wholegrain listed as the first ingredient on the food label</td>
<td>refined grain products (&lt; 0.2 servings of WG foods)</td>
<td>no data</td>
<td>no difference in insulin sensitivity⁸, fasting and 2-hour glucose and insulin concentrations, and glucose and insulin AUCs in OGTT</td>
</tr>
<tr>
<td></td>
<td>metabolic syndrome⁷</td>
<td></td>
<td>approximately 5 servings/d</td>
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<tr>
<td></td>
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<td></td>
<td>1 serving is 1 slice of bread, or 28 g of ready-to-eat cereal, or 120 ml of cooked cereal/rice/pasta</td>
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</tbody>
</table>

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<table>
<thead>
<tr>
<th>Year of study, country (reference)</th>
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<th>Study design</th>
<th>Wholegrain products (intake/d)</th>
<th>Refined grain products (intake/d)</th>
<th>Intake of wholegrain ingredients/d</th>
<th>Results¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>2010, UK (83)</td>
<td>266 (approximately 50% women); 46 ± 10 y; 30 ± 4 kg/m² (median ± SD) healthy</td>
<td>randomized parallel; 16 wk (2 treatment groups and 1 control group)</td>
<td>subjects freely selected from provided foods: wholewheat bread, shredded wheat, cheerios, porridge oats, brown basmati rice, wholewheat pasta, Weetabix, oat bar, wholegrain crisps (in all products content of wholegrains was &gt; 50% except rice and pasta)</td>
<td>subjects continued their habitual consumption of a low wholegrain diet (consumption of refined grain foods was not controlled)</td>
<td>74 ± 28.5 g (group 1); 83 ± 31.1 g at wk 8 and 115 ± 39.6 g at wk 16 (group 2) in control group: 19 ± 19.9 g (mean ± SD)</td>
<td>no difference in insulin sensitivity⁹, and fasting glucose and insulin concentrations</td>
</tr>
<tr>
<td>2010, Italy (84)</td>
<td>3 women, 12 men; 55 ± 8 y; 27 ± 3.0 kg/m² (mean ± SD) healthy</td>
<td>randomized cross-over; 2 x 3 wk</td>
<td>wholemeal wheat bread, pasta, rusks, crackers</td>
<td>the same products in refined form</td>
<td>no data</td>
<td>no difference in insulin sensitivity⁶ and fasting glucose and insulin concentrations</td>
</tr>
<tr>
<td>2010, Sweden (88)</td>
<td>17 men, 73.5 ± 4.6 y, 27.5 ± 4.6 kg/m² (mean ± SD) prostate cancer</td>
<td>randomized crossover; 2 x 6 wk</td>
<td>wholegrain rye and rye bran products: 247 ± 34 g of bread, 89 ± 17 g of crisp bread, 50 ± 9 g of breakfast cereals, 35 ± 10 g of porridge (uncooked) (mean ± SD)</td>
<td>wheat products with added cellulose: 245 ± 39 g bread, 96 ± 19 g crisp bread, 39 ± 15 g breakfast cereals, 29 ± 5 g porridge (uncooked) (mean ± SD)</td>
<td>no data</td>
<td>lower fasting insulin concentration, no difference in fasting glucose</td>
</tr>
<tr>
<td>2010, UK (87)</td>
<td>102 women, 104 men; 52 ± 1 y; 28 ± 0.5 kg/m² (mean ± SEM) Healthy, or features of metabolic syndrome or moderate hypercholesterolemia</td>
<td>randomized parallel; 16 wk</td>
<td>group 1: 3 servings of whole wheat foods (70-80 g wholemeal bread and 30-40 g wholegrain cereals)</td>
<td>refined grain foods</td>
<td>no data</td>
<td>no difference in insulin sensitivity⁶,⁸ and fasting glucose and insulin concentrations</td>
</tr>
</tbody>
</table>

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<thead>
<tr>
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<th>Refined grain products (intake/d)</th>
<th>Intake of wholegrain ingredients/d</th>
<th>Results¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>2011, Finland (86)¹⁰</td>
<td>35 men, 34 women; 58 ± 8 y (WGED group)/59 ± 7 (refined grain group), 31.4 ± 3.4 kg/m² (WGED group)/31.0 ± 3.6 kg/m² (refined grain group) (mean ± SD)</td>
<td>randomized parallel; 12 wk</td>
<td>7.9 portions of the test breads: 50% share of wholegrain rye breads, 40% share of endosperm rye bread, 10% share of sourdough wholemeal wheat bread wholemeal pasta 3.5 dl/wk (uncooked), voluntary intake of wholegrain oat biscuit</td>
<td>6.8 portions of white wheat bread advice to consume low fibre porridge and pasta (maximum of 1-2 portions/d of rye products allowed)</td>
<td>no data</td>
<td>no difference in insulin sensitivity⁶,⁹, fasting glucose and insulin concentrations, 2-hour glucose and insulin concentrations, glucose and insulin AUCs in OGTT, and indexes of first-phase insulin secretion in OGTT (IGI and DI) trend for decreased 2-hour glucose concentration within WGED group</td>
</tr>
<tr>
<td>2011, Switzerland (96)</td>
<td>6 men, 11 women men, 36.5 ± 4.2 y, 24.5 ± 0.6 kg/m²; women, 34.1 ± 3.0 y, 23.2 ± 0.9 kg/m² (mean ± SEM)</td>
<td>randomized crossover; 2 x 2 wk</td>
<td>breakfast cereal, bread, crackers, cereal bar, pasta, rice, couscous; 64% of wheat, 13% of oats, 9% of brown rice, the rest from barley and rye</td>
<td>breakfast cereal, bread, crackers, cereal bar, pasta, rice, couscous; 66% wheat, 27% rice, 8% maize</td>
<td>151 ± 15 g (mean ± SD)</td>
<td>no difference in fasting glucose concentration 0 g from the refined grain diet</td>
</tr>
</tbody>
</table>

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<table>
<thead>
<tr>
<th>Year of study, country (reference)</th>
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<th>Wholegrain products (intake/d)</th>
<th>Refined grain products (intake/d)</th>
<th>Intake of wholegrain ingredients/d</th>
<th>Results¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>2012, Canada (91)</td>
<td>two groups of subjects:</td>
<td>randomized crossover; 2 x 6 wk</td>
<td>wholegrain wheat sourdough bread: 150 g for women, and 175 g for men</td>
<td>white wheat bread; 150 g for women and 188 g for men</td>
<td>no data</td>
<td>no differences in fasting glucose and insulin concentrations</td>
</tr>
<tr>
<td></td>
<td>10 men, 4 women; 53 ± 6 y; 26.5 ± 2.9 kg/m²; normal fasting and 2-hour glucose concentrations, normal fasting insulin concentration</td>
<td>(subjects with normal and elevated glucose and/or insulin were studied separately)</td>
<td></td>
<td></td>
<td>lower 2-hour glucose concentration and glucose AUC in OGTT in subjects with IFG/IGT/elevated fasting insulin</td>
<td>higher IGI in both groups of subjects (a trend among subjects with IFG/IGT/elevated insulin), a trend for lower 2-hour insulin concentration in subjects with IFG/IGT/elevated fasting insulin</td>
</tr>
<tr>
<td></td>
<td>10 men, 4 women; 57 ± 7 y; 35.7 ± 5.7 kg/m²; IFG or IGT or elevated fasting insulin concentration (≥ 90 pmol/L) but no diabetes</td>
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</table>

¹ refined grain diet as reference; ² the rye breads consumed contain 94%, 82%, 54% and 36% of wholegrain by weight (Vaasan crisp bread, Oululainen Jälkiununleipä, Real, and Vaasan ruispalat, respectively); ³ insulin sensitivity measured by euglycemic hyperinsulinemic clamp technique; ⁴ insulin sensitivity and acute insulin response measured by frequently sampled intravenous glucose tolerance test (FSIGT); ⁵ diets were hypocaloric; weight lost is adjusted in the results; ⁶ insulin sensitivity calculated from fasting values using homeostatic model assessment of insulin resistance (HOMA-IR); ⁷ according to National Cholesterol Education Program Adult Treatment Panel III criteria (77); ⁸ insulin sensitivity calculated from fasting values using revised/modified quantitative insulin sensitivity check index (QUICKI); ⁹ insulin sensitivity calculated from fasting values using revised/modified quantitative insulin sensitivity check index (QUICKI); ¹⁰ a third study arm was included in the intervention and statistical analyses; AUC, area under the curve; DI, disposition index; IFG, impaired fasting glucose; IGI, insulinogenic index; IGT, impaired glucose tolerance; OGTT, oral glucose tolerance test; WGED, wholegrain enriched diet
Postprandial studies

Increase in plasma concentrations of glucose and insulin after ingestion of a meal containing carbohydrates is tightly regulated but may vary according to the source of carbohydrates. Increase in postprandial glucose concentration over the first few hours after a meal leads to elevated secretion of insulin and a subsequent steep decrease in glucose concentration even below the initial fasting level (97). The sharp fall is balanced by counter-regulatory hormones, which also increase the concentration of free fatty acids in circulation. Hyperinsulinemia (that is, high concentration of insulin in blood) and high concentration of free fatty acids may cause insulin resistance (97,98). Repeated consumption of meals which first result in a rapid increase in blood glucose and insulin followed by a steep decrease in glucose may thus cause insulin resistance, which in turn triggers the pancreatic beta cells to secrete more insulin (97). In the long run, the optimal function of these beta cells may be disturbed, leading to disturbances in glucose homeostasis and increased risk of type 2 diabetes. On the contrary, consumption of carbohydrate-containing meals which lead to a moderate increase in glucose and/or insulin concentrations may preserve beta cell function.

GI is defined as the incremental glucose area under the curve (AUC) after ingestion of a standard amount of carbohydrate from a test food relative to that of a control food (either white wheat bread or glucose) (99). However, GI is measured in healthy individuals only over the first two hours after a test food, although metabolic events due to ingestion of a meal occur over a longer postprandial period (97). Furthermore, GI or the calculated glucose AUC does not take into account the form of the glucose curve. The same GI values or glucose AUC values can be found in two different physiological conditions: when glucose concentration quickly increases to a high level and decreases below the fasting level as quickly, and when glucose concentration slowly increases to a moderate level and then slowly decreases, staying above fasting level for a longer period of time. Thus, describing the form of the glucose curve might be more relevant than only reporting GI or glucose AUC. To describe the form of the glucose curve, one can report glucose concentrations at different time points after a meal, or give incremental glucose peaks, or calculate an index suggested to portray the glycaemic profile, GP (100). Insulinaemic index (II), defined similarly to GI but using the corresponding insulin values, describes the insulin response after a meal. It has been shown that GI and II are not always in agreement, i.e. a food producing low GI can produce high II (101). Thus, determining both glucose and insulin responses provides a more complete picture of postprandial glucose metabolism.

Several studies have investigated the effects of intake of wheat and rye breads as compared to that of white wheat bread on postprandial glucose and insulin responses in healthy subjects (Table 2.6). To summarize these studies, breads with large amounts of intact wheat or rye kernels reduce postprandial glucose and insulin responses. However, the beneficial effects of whole kernels in wheat bread are lost when the grains are milled to flour (102-104). Thus, it is unlikely that glycaemic response of conventional wholegrain foods made of wholemeal wheat flour would be a reason for a decreased risk of type 2 diabetes, although this hypothesis is suggested in some of the prospective cohort studies.

Ingestion of rye breads, whether made of wholegrain, wholemeal or endosperm flour, reduces postprandial insulin response even without affecting the glucose response (Table
2.6). However, white wheat bread enriched with native rye bran does not change the insulin response from that induced by white wheat bread (100). Moreover, the insulin response to rye bread is reduced both with and without using sourdough fermentation in baking the bread and independently of the acidity of the bread (61,62,100,105-107). Thus, wholegrain and endosperm rye breads have a specific lowering effect on the postprandial insulin response, although the glucose response is not necessarily affected. However, the postprandial glucose curve after intake of rye bread seems to be more beneficial; that is, the incremental peak in glucose concentration is lower and/or glucose concentration in the late postprandial phase (at two hours or later) stays higher as compared to that after white wheat bread (62,100,107). Rye bread produces a beneficial GP associated with reduced postprandial insulin response (100,107). In a recent study, the GP of rye bread was not associated with reduced insulin response, but the authors explained this by the structure of the rye breads, which resembled porridge more than bread (108).
<table>
<thead>
<tr>
<th>Year of study, (reference)</th>
<th>Subjects (n)</th>
<th>Test breads¹</th>
<th>Results²</th>
</tr>
</thead>
<tbody>
<tr>
<td>1992 (109)</td>
<td>3 men, 7 women</td>
<td>whole kernel wheat bread (containing 80%</td>
<td>glucose concentrations lower at 45 and 70 min, lower GI³, insulin concentrations lower at 45, 95, and 120 min, lower II³</td>
</tr>
<tr>
<td></td>
<td></td>
<td>kemels and 20% white wheat flour)</td>
<td></td>
</tr>
<tr>
<td>1992 (110)</td>
<td>5 men, 5 women</td>
<td>wheat kernel bread and rye kernel bread (containing 80% kemels and 20% white wheat flour)</td>
<td>glucose concentrations lower for wheat kernel bread at 45, 70, and 95 min; and for rye kernel bread at 30, 45, and 70 min; glucose concentrations higher for both breads at 180 min, lower GI³ for both breads</td>
</tr>
<tr>
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<tr>
<td>1994 (111)</td>
<td>4 men, 3 women</td>
<td>pumpernickel bread (containing rye kernels and white wheat flour)</td>
<td>glucose concentrations lower at 45 and 70 min, lower GI³, insulin concentrations lower at 45 and 95 min, lower II³</td>
</tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>1999 (102)</td>
<td>13 men, 13 women</td>
<td>wholegrain wheat bread (coarsely ground kemels, particle size of flour &lt; 850 μm with 50% &lt; 150 μm) and wholemeal wheat bread (finely ground kemels, particle size of flour &lt; 150 μm)</td>
<td>glucose concentration lower for both breads at 60 min, no difference in 2-hour glucose AUC</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>no difference in insulin concentrations and 2-hour insulin AUC</td>
</tr>
<tr>
<td>1999 (106)</td>
<td>10 men, 10 women</td>
<td>wholemeal rye bread with whole kernels (baked with sourdough fermentation)</td>
<td>no differences in glucose responses, insulin concentrations lower at 45, 60, 90, 120, and 150 min, lower insulin AUC and maximal insulin response</td>
</tr>
<tr>
<td>2002 (105)</td>
<td>10 men, 10 women</td>
<td>wholekernel rye bread (60% whole rye kemels, 40% rye flour) with added lactic acid</td>
<td>no difference in glucose AUC, insulin concentrations lower at 30, 45, 60, 90, 120, and 150 min, lower maximal insulin response and insulin AUC</td>
</tr>
<tr>
<td>2003 (62)</td>
<td>19 postmenopausal women</td>
<td>wholemeal rye bread, endosperm rye bread, and wholemeal rye bread enriched with rye bran (all baked with sourdough fermentation)</td>
<td>higher glucose concentrations for endosperm and wholemeal rye breads at 150 and 180 min, no other differences in glucose responses</td>
</tr>
</tbody>
</table>

(Table continues on the next page)
<table>
<thead>
<tr>
<th>Reference</th>
<th>Subjects (n)</th>
<th>Test breads¹</th>
<th>Results²</th>
</tr>
</thead>
<tbody>
<tr>
<td>2009 (104)</td>
<td>10 men</td>
<td>wholemeal wheat bread</td>
<td>no difference in glucose responses and 3-hour glucose AUC no difference in insulin responses and 3-hour insulin AUC</td>
</tr>
<tr>
<td>2009 (112)</td>
<td>3 men, 7 women</td>
<td>wholemeal rye bread with whole kernels</td>
<td>no difference in glucose responses insulin concentrations were not measured</td>
</tr>
<tr>
<td>2009 (100)</td>
<td>9 men, 3 women</td>
<td>endosperm rye bread (containing 75 and 25% of endosperm rye and white wheat flour, respectively), wholegrain rye bread (containing 75 and 25% of wholegrain rye and white wheat flour, respectively), wholegrain rye bread with added lactic acid, rye bran bread (containing 65 and 35% of white wheat and rye bran flour, respectively)</td>
<td>lower GI³ and higher Gp⁴ (i.e. more beneficial form of glucose curve) for endosperm rye bread and wholegrain rye bread with lactic acid lower II³ for endosperm rye bread, wholegrain rye bread, and wholegrain rye bread with lactic acid</td>
</tr>
<tr>
<td>2010 (103)</td>
<td>6 men, 10 women</td>
<td>wholemeal wheat bread</td>
<td>no difference in glucose responses insulin concentrations were not measured</td>
</tr>
<tr>
<td>2011 (107)</td>
<td>5 men, 5 women</td>
<td>endosperm rye bread, endosperm rye bread with added lactic acid, wholegrain rye bread (made of coarse rye flour), wholegrain rye bread with added lactic acid</td>
<td>lower GI³ and incremental glucose peak for endosperm rye bread with lactic acid and for both wholegrain rye breads, no differences in Gp⁴ lower II³ and incremental insulin peak for both endosperm rye breads and both wholegrain rye breads</td>
</tr>
<tr>
<td>2011 (108)</td>
<td>7 men, 7 women</td>
<td>five rye breads (made of 100% wholegrain rye flour), each from a different rye variety (Amilo, Nikita, D. Zlote, H. Loire, Rekrut)</td>
<td>no differences in glucose responses lower II³ and 3-h insulin AUC for wholegrain rye breads from Amilo and Rekrut, lower incremental insulin peak for rye bread from Amilo, no differences in insulin concentrations over 180 min</td>
</tr>
</tbody>
</table>

(Table continues on the next page)
Table 2.6. (continued)

<table>
<thead>
<tr>
<th>Reference</th>
<th>Subjects (n)</th>
<th>Test breads&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Results&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>2011 (113)</td>
<td>10 men, 10 women</td>
<td>six rye breads (75% wholegrain rye flour, 25% white wheat flour); five from a different rye variety (Vicello, Picasso, Kaskelott, Amilo, Evolo) and one from a commercial Swedish wholegrain rye blend</td>
<td>lower GI&lt;sup&gt;3&lt;/sup&gt; for Picasso and Vicello; lower incremental glucose peak for Picasso, Vicello, Amilo, and Evolo; no differences in GP&lt;sup&gt;4&lt;/sup&gt;; no differences in 3-hour glucose AUC</td>
</tr>
<tr>
<td>2011 (61)</td>
<td>3 men, 13 women</td>
<td>sourdough endosperm rye bread</td>
<td>higher glucose concentration at 90 min, no difference in glucose AUC and incremental glucose peak</td>
</tr>
</tbody>
</table>

<sup>1</sup> Ingested portion of test and control breads included 50 g of available carbohydrates, except for Behall et al. (1999), Leinonen et al. (1999), Hlebowicz et al. (2009), and Rosén et al. (2009); <sup>2</sup> white wheat bread as a control; <sup>3</sup> GI and II calculated from 2-hour glucose and insulin areas under the curve, respectively, using white wheat bread as reference; <sup>4</sup> GP calculated as the time during which blood glucose is above fasting concentration divided by the incremental peak value of blood glucose; AUC, area under the curve; GI, glycaemic index; GP, glycaemic profile; II, insulinaemic index
Although sourdough fermentation does not seem to explain the effects of rye bread on glucose and insulin responses, using this method in baking white wheat bread or wholemeal wheat bread seems to result in a beneficial effect on postprandial glucose and/or insulin responses as compared to similar breads baked with straight dough (i.e. leavening with yeast) (104,114-116). It is not known why sourdough fermentation of wheat bread decreases the postprandial responses. Sourdough fermentation in baking wholemeal barley bread or addition of lactic acid into bread, or of acetic acid as vinegar in bread meal, the main organic acids produced by microbes in the sourdough, reduced postprandial glucose and insulin responses (111,117-120). The authors suggested that the effect was due to a delayed rate of starch digestion and delayed gastric emptying rate by lactic acid and acetic acid, respectively, either added in the bread or produced by sourdough fermentation. An in vitro study supports the role of lactic acid in delaying starch digestion by showing that lactic acid may promote interactions between wheat starch and gluten during heat treatment (such as in bread baking), which reduces availability of starch for digestion (121). However, in one study, the in vitro rate of starch digestion did not explain differences in glucose release between sourdough and non-sourdough wheat breads (116), and the authors of another study suggested that a more tense and rigid texture of sourdough-fermented wheat bread might decrease digestion of starch (115).

It is not known why a meal containing rye bread reduces postprandial insulin response. the gastric emptying rate was not reduced after ingestion of rye bread as compared to white wheat bread, regardless of the method used to measure the rate (61,105,112). Properties or component(s) of endosperm rye flour (100) or decreased starch hydrolysis due to the tructure of rye bread (62,105) are possible mechanisms which have been suggested to reduce the insulin response.

2.3 EFFECTS OF WHOLEGRAIN WHEAT AND RYE IN INTESTINAL TRACT ON GLUCOSE METABOLISM

2.3.1 Intestinal microbiota composition
The intestinal microbiota is a densely populated, complex, and metabolically active ecosystem. There are $10^{14}$ microbes residing in the human gut, the majority colonizing the large intestine (122). All the microorganisms (including bacteria, archaea, yeasts, and fungi) residing in the human intestinal tract can be called intestinal microbiota (123). Human bacterial microbiota consists of nine phyla (Firmicutes, Bacteroidetes, Actinobacteria, Fusobacteria, Proteobacteria, Verrucomicrobia, Cyanobacteria, Spirochaetes, and VadinBE97) (122) of which Firmicutes and Bacteroidetes dominate accounting for > 90% of all defined phylotypes in healthy humans (124). A phylotype is defined as a cluster of related 16S rRNA gene sequences which share ≥ 97% identity (122,125). The phyla in turn are divided into lower phylogenetic levels: class, order, family, genus, phylotype (i.e. species-level), and strain. Genomics-based techniques sequencing microbial 16S rRNA genes have enabled more comprehensive identification of the microbial diversity than traditional culture-based methods. Almost 2000 distinct uncultured bacterial phylotypes were reported to be found in the human gastrointestinal tract by 2008, while the number of cultured bacterial species was approximately 400 (124).
Intestinal microbiota is highly individual at the phylotype level and temporally stable after early childhood (122,125). The subject-specificity of the microbiota seems to remain even over eight to twelve years of follow-up of healthy individuals (126). Despite the normally large inter-individual variation in the composition of intestinal microbiota, clear differences in the composition are observed between healthy and diseased individuals. Not only disturbances in intestinal health (i.e. inflammatory bowel diseases and irritable bowel syndrome) but also obesity and type 2 diabetes have been associated with altered composition of intestinal microbiota (125). At the phylum level, diabetics had lower proportion of Firmicutes than non-diabetics, but there were no differences in the microbiota composition at the genus level (127). Another study observed that the proportion of the genus *Bacteroides* was higher and number of *Bifidobacterium* lower in subjects with diabetes than in those without diabetes (128). These two studies were conducted with only less than twenty diabetic or control subjects. A study with a larger sample size of Chinese individuals reported that occurrence of butyrate-producing bacteria was lower in subjects with type 2 diabetes than in healthy controls (129). Furthermore, there is one study showing a greater amount of the *Eubacterium rectale-Clostridium coccoides* bacterial group, but no difference in other groups such as *Bifidobacterium* and *Bacteroides*, in women with metabolic syndrome than in those without metabolic syndrome (130). Instead, in obese men with metabolic syndrome, it was reported that faecal content of Bacteroidetes (including *Bacteroides* genus) is higher and that of *Clostridium* cluster XIVa (including *Eubacterium rectale*) lower as compared to the content of the same bacterial groups in lean, healthy men (131). These few case-control studies, although with somewhat inconsistent results, indicate that individuals with type 2 diabetes or metabolic syndrome have differences in the composition of their intestinal microbiota from that of healthy individuals. However, it is not known whether the observed changes in the intestinal microbiota composition would increase the risk of type 2 diabetes, or whether the disease per se changes the composition. It is of interest, however, that recently a faecal transplantation from lean, healthy men into the small intestine of men with the metabolic syndrome improved insulin sensitivity (measured by the hyperinsulinenic euglycemic clamp), while also increasing the proportion of butyrate-producing *Roseburia intestinalis* and other Firmicutes in the faeces and butyrate-producing *Eubacterium hallii* in small intestinal biopsies (131). The improved insulin sensitivity took place without any change in body weight or energy intake.

The possible link of intestinal microbiota composition to disturbances in glucose metabolism and other metabolic conditions has been reviewed widely in recent years, mainly based on studies in rodents. Among others, Cani et al. (2008) have reviewed a series of mice studies and suggest that feeding a high-fat diet to mice increases absorption of lipopolysaccharide (LPS), a cell membrane component of gram-negative bacteria, from the intestine into peripheral circulation (132). Absorption of LPS may be facilitated due to increased gut permeability (133). LPS in circulation induces low-grade inflammation characterized by an increased level of several cytokines (132), which in turn induces insulin resistance (134). In humans, it was observed that the concentration of serum LPS was higher in subjects with type 2 diabetes than in those without type 2 diabetes, and the concentration of fasting insulin correlated positively with that of LPS in non-diabetic subjects (135). Total energy intake, but not fat intake, correlated positively with plasma
concentration of LPS in healthy men (136), suggesting that excess intake of energy in humans might contribute to increased concentration of LPS in circulation.

Non-digestible carbohydrates are degraded and used for growth by the intestinal microbiota of humans (137). Low-carbohydrate diets (4-5 E% from carbohydrates, 6-13 g/d of fibre) as compared to normal diets (22-32 E% from carbohydrates, 22-32 g/d of fibre) have been shown to decrease numbers and proportions of *Roseburia* and *Eubacterium rectale* bacterial groups, which are butyrate-producers, and bifidobacteria (138-140). The decrease in *Roseburia* and *Eubacterium rectale* groups was greater with greater reduction in carbohydrate intake. These studies with low-carbohydrate diet show potential detrimental effects on the intestinal microbiota composition as butyrate producers and bifidobacteria decreased. However, it is not clear whether the effects were due to the reduced intake of total carbohydrates or due to the reduced intake of dietary fibre because, along with decreasing the intake of carbohydrates, the intake of non-starch polysaccharides, or fibre, decreased. Thus, it is relevant to compare diets with a similar content of carbohydrates but different contents of wholegrains or grain fibre to clarify the effect of grain-based diet on intestinal microbiota composition.

*Effects of grain-based diets on intestinal microbiota composition*

Although wholegrains include several components such as oligofructose and arabinoyxlan, which can be fractionated and are available as commercial supplements, only wholegrain foods and grain fibre in their natural form (i.e. whole foods) are focused on here because they are the foods consumed in the prospective cohort and intervention studies referred to earlier. In humans, there is only one intervention study (96) comparing a diet based on wholegrain foods with that based on refined grain foods, while other studies have used variable test and control diets containing grain fibre. The composition of intestinal microbiota has been analysed from faeces because they are the only practical samples available from humans for this purpose. A two-week wholegrain diet, in which wholegrain foods were composed mainly of wheat (64%) and to a smaller extent of oats, brown rice, barley, and rye, consumed by healthy subjects with habitually low wholegrain intake increased numbers of *Clostridium leptum* as compared to a refined grain diet, but did not affect any other analysed bacterial group (96).

Also, the studies with variable grain-containing test and control diets show little or no effect on microbiota composition. A three-week consumption of a wholegrain wheat or wholegrain maize breakfast cereals increased numbers of faecal bifidobacteria in normal and overweight subjects as compared to consumption of control cereals which were composed of wheat bran or refined grain maize (141,142). Wholegrain wheat cereal also increased the numbers of the lactobacilli/enterococci group more than wheat bran cereal (142). A three-week consumption of a diet supplemented with wheat bran and providing 42 g/d of non-starch polysaccharides as compared to a control diet without added wheat bran did not affect the analysed composition of the intestinal microbiota in men with metabolic syndrome (143). The control diet provided 28 g/d of non-starch polysaccharides which is already a high amount and might explain why there was no difference in the microbiota composition between the two diets. Consumption of rye bran by healthy postmenopausal women habitually consuming rye as compared to that of inert wheat cellulose providing a
similar amount of fibre (14 g/d) for six weeks did not change bacterial composition (144). In another study, when the composition of the faecal microbiota was analysed with a culture-dependent method, an eight-week daily consumption of wholegrain rye bread enriched with rye bran was not observed to change microbiota composition as compared to that of white wheat bread in Finnish postmenopausal women (145). When normal weight and overweight subjects with normal fasting glucose concentration consumed wholegrain barley flakes (60 g/d providing 19 g/d fibre) daily for four weeks as compared to brown rice (60 g/d providing 4 g/d fibre), proportions of genera Roseburia, Bifidobacterium, and Dialister increased (146). No other differences in microbiota composition between the diets were observed at genus or phylum level. Finally, in an intervention study with overweight or obese subjects with features of the metabolic syndrome, the diet of the subjects was supplemented with insoluble cereal fibre extract (purified from oat hulls) providing 43 g of cereal fibre daily whereas the control diet, without supplementation, provided 14 g of cereal fibre daily (147). Despite the three-fold difference in the intake of cereal fibre, the diets did not show differences in the proportions of dominant bacterial groups after six or 18 weeks of consumption.

All of the above-mentioned human intervention studies were conducted with targeted methods which analyse specific, pre-determined, dominant or otherwise relevant bacterial groups (except for Martinez et al. (146), who used pyrosequencing). All the studies also followed cross-over design, except for the intervention study by Weickert et al. (147), which was a parallel study. These few studies suggest that wholegrains may have a different effect on intestinal microbiota composition from that of refined grains, that the effect of wholegrains may be stronger than that of bran, and that different varieties of wholegrains with different amounts of fibre may vary in their effects on microbiota composition. Wholegrain-derived changes in microbiota composition may, based on the theory derived from the animal studies, affect glucose metabolism in humans.

### 2.3.2 Phenolic acid metabolism

Phenolic acids are a group of phenolic compounds of which ferulic acid (FA), sinapic acid, and p-coumaric acid are the ones existing in the highest amount in wheat and rye (see section 2.1.3; p. 10). Phenolic acids, among other phytochemicals, are suggested to mediate the health effects of wholegrains (50,148). Based on in vitro studies, free phenolic acids have a high antioxidative and anti-inflammatory activity (50). Low-grade inflammation has a fundamental role in contributing to insulin resistance and beta-cell dysfunction, which may in turn lead to the development of type 2 diabetes (149). Anti-inflammatory phenolic acids in wholegrains might thus have properties which prevent disturbances in glucose metabolism. However, in vitro studies do not necessarily imply that phenolic acids in grains would have similar in vivo effects in peripheral tissues of humans, because the original fibre-bound phenolic acids may not reach the tissues.

FA is clearly the most abundant one of the phenolic acids in wheat and rye (150), and 0.2% and 1.3% of the total FA, respectively, was shown to exist in a free form (55,151). Most probably, this free form of FA in wheat bran was absorbed in the small intestine of humans as concluded from postprandially elevated concentrations of FA in plasma (152,153). Ingestion of wheat bran also increased excretion of FA and other phenolic acids in urine
over four or twenty-four hours follow-up after a single meal (152,154). However, the excreted amount in urine was only 3-4% of the FA ingested from native wheat (152,153). There are no human studies of postprandial absorption of FA in rye, but there is one study showing that regular consumption of rye bran increased urinary excretion of FA (155).

As seen in the posprandial studies, FA in grains is mainly not unavailable for absorption in the small intestine. This is because most of the FA is bound to arabinoxylan through an ester linkage (156). The bound FA is delivered with fibre into the large intestine. In the large intestine, xylanases and esterases from intestinal microbiota release FA from the fibre complex (157,158). After being released, FA is rapidly converted to metabolites by intestinal microbiota (159-161). Because they are absorbed from the large intestine into circulation, these metabolites are observed in human urine and blood (153,162). Indeed, a two-fold higher concentration of some colon-derived phenolic acid metabolites was detected in urine after a three-week ingestion of wheat bran or wholegrain wheat as compared to a diet without bran or wholegrains (142). After absorption the metabolites, as well as the parent compounds, undergo further metabolism in the liver, and might be involved in entero-hepatic circulation, before excretion in urine (163). The part that is not absorbed and excreted in urine is finally excreted in faeces.

Because of the delivery of the phenolic acids into the large intestine, subsequent release from the fibre complex by intestinal microbiota and absorption into circulation, regular consumption of wholegrain foods and foods rich in grain fibre has been suggested to contribute to the health benefits of wholegrains (53).

2.3.3 Short chain fatty acids
Short chain fatty acids (SCFAs) acetate, propionate, and butyrate are produced from indigestible carbohydrates in intestinal fermentation by the microbiota (164). When analysed from faeces, acetate is the main SCFA produced, followed by propionate and butyrate in molar ratios of approximately 60:20:20. Fermentation mainly takes place in the proximal large intestine because it is the site with the highest amount of diet-derived substrates available for fermentation after escaping absorption in the small intestine. SCFAs are rapidly absorbed from the intestine, but a small proportion is also excreted in faeces. Most of the butyrate is metabolized by the intestinal epithelium cells, whereas the main sites for metabolism are the liver and the muscles for acetate and the liver for propionate. (164).

It has been suggested based on animal and in vitro studies that circulating acetate and propionate absorbed from the intestine inhibit release of free fatty acids from adipocytes, reduce adipocyte cell size, and decrease low-grade inflammation, which in turn improves insulin sensitivity of tissues (165). Meijer et al. (166) have also presented possible mechanisms based on in vitro studies and suggested that SCFAs, especially butyrate, affect immune cell function leading to anti-inflammatory effects. Glucose metabolism after ingestion of grain products has been investigated in overnight second-meal studies with simultaneous determination of plasma concentration of SCFAs (167-171). In the second-meal studies, evening meals rich vs. low in indigestible carbohydrates from grains were ingested by healthy subjects ten to twelve hours before a standardized low-fibre breakfast,
or OGTT in the study by Priebe et al. (170), and the effects on glucose metabolism and/or plasma concentration of SCFAs were studied in fasting and postprandial states the following morning.

First, postprandial glucose response to a standardized test meal on the following morning was reduced after ingestion of boiled barley kernels or breads with a high amount of barley kernels as compared to that of white wheat bread (167,168,170,172,173). Postprandial insulin response also was found to be reduced in some but not all of the studies (167,168,170,172). Along with the improved glucose response, two studies reported increased fasting and postprandial plasma butyrate concentration on following morning after barley kernel evening meals as compared to the white wheat bread evening meal, while there was no difference in acetate and propionate concentrations (169,170). Instead, one study reported a higher mean concentration of plasma propionate (calculated from fasting and three-hour postprandial concentrations) on following morning after the barley kernel evening meal than after the white wheat bread evening meal, while there was no difference in acetate and butyrate concentration (167). Nilsson et al. (169) correlated SCFAs and glucose responses and observed that plasma concentrations of acetate, and especially butyrate, were positively correlated with the content of indigestible carbohydrates of the evening meals, and acetate and butyrate concentrations were inversely associated with postprandial glucose response. Furthermore, Priebe et al. (170) measured the glucose clearance rate, i.e. uptake of glucose into peripheral tissues, and peripheral insulin sensitivity, and observed that the former was higher and the latter improved the morning after an evening meal containing barley kernels as compared to white wheat bread.

These studies suggest that indigestible carbohydrates in barley may contribute to the production of SCFAs, especially propionate and butyrate, which may have beneficial effects on postprandial glucose metabolism in an overnight perspective. However, when subjects ingested a pasta meal with added barley fibre or barley flour porridge the previous evening, postprandial glucose and insulin responses were not improved the following morning (174). Instead, white wheat bread enriched with resistant starch and barley fibre improved postprandial glucose response the following morning (168). This suggests that the preserved botanical structure of barley kernels or resistant starch in combination with barley fibre is needed to improve glucose metabolism in an overnight perspective.

As for wholegrain wheat or wheat fibre, ingestion of an evening meal containing wholemeal wheat bread, with 3.6-fold more fibre than a control meal containing white wheat bread, did not change glucose response to a standardized breakfast the following morning (175). Furthermore, both a low-fibre pasta evening meal with added wheat bran providing four-fold more fibre than white wheat bread meal, and wheat kernels providing the same amount of fibre as barley kernels, produced similar postprandial glucose and insulin responses to a white wheat bread evening meal the following morning (167). These results suggest that unlike barley kernels, wholemeal wheat bread, wheat bran, and wheat kernels are not effective in improving postprandial glucose metabolism in an overnight perspective. However, ingestion of white wheat bread enriched with purified, insoluble wheat or oat fibre as compared to that of white wheat bread in three doses over the previous day decreased the three-hour glucose AUC the following morning after a
standardized breakfast (171). No differences were observed in insulin responses, or maximal glucose concentration between the treatments. This study implies that wheat fibre might nevertheless affect glucose metabolism, if not in an overnight perspective, probably when ingested over a longer period of time. The authors (171) concluded that intestinal fermentation did not contribute to the reduced glucose response because the concentration of fasting serum butyrate was not different the morning after the days the different breads were ingested. However, postprandial SCFA concentrations in plasma were not measured. Nilsson et al. (169) observed increased propionate and butyrate concentrations in plasma postprandially at 30 min as compared to fasting state, which may suggest that the fasting state is not always informative enough for investigating intestinal fermentation.

There are no second-meal studies with rye consumption involved. Because proportions of fibre components in rye differ from those in wheat (see section 2.1.3; p. 9), and as rye bread has a different postprandial insulin response from that of wheat bread (see section 2.2.3, Postprandial studies; p. 20-21), it could be hypothesized that ingestion of rye bread the previous day might have influence glucose metabolism the following morning.

Longer-term ingestion of a diet based on high-fibre rye bread as compared to one on low-fibre white wheat bread has been observed to increase faecal concentration of butyrate in men but not in women (95,176). Even though the daily intake of fibre from rye breads was 12 g more in postmenopausal women (145) than in the men of the previous study (176), consumption of rye breads enriched with rye bran did not increase the faecal concentration of butyrate as compared to that of white wheat bread in postmenopausal women (145). Faecal acetate and propionate concentrations did not differ between the bread periods (95,145,176).

It should be noted that faecal concentrations of SCFA represent only a small proportion of the produced SCFA because 90-95% of the SCFA is quickly absorbed in the caecum and colon (164). Thus, investigating faecal SCFA does not provide an accurate way to measure SCFA production. In fact, there is no in vivo method in humans to study the production of SCFAs in the large intestine. The only reasonable method to estimate this production is to measure the peripheral blood concentrations of SCFA. Bach Knudsen et al (177) concluded in their review that in pigs, increased intestinal production of butyrate raises the circulating concentration of butyrate. In humans, blood concentrations of SCFAs after rye intake have been reported previously only once (177). As compared to a low-fibre diet based on white wheat bread, a high-fibre rye bread diet (26 g/d and 31 g/d of fibre from the rye breads in women and men, respectively) did not affect the fasting concentration of total SCFA or butyrate in venous blood in Finnish men and women.

Cannulated pigs have been used as a model to study the portal and peripheral blood concentrations of SCFA. Feeding rye bread to pigs has been reported to increase the concentration of butyrate in portal and peripheral blood as compared to feeding wheat bread or wheat bread enriched with purified insoluble wheat fibre (177,178). However, feeding of rye bread did not change postprandial glucose and insulin responses in pigs (178). Another study reported a lowered insulin response due to ingestion of bread containing rye aleurone as compared to that of wholegrain wheat bread, and showed that
the reduced insulin response was associated with increased production of butyrate (179). It was also noted that, in humans, the postprandial state might be more relevant to observe changes in portal SCFA concentration than the fasting state, based on observations in sudden-death victims and patients undergoing surgery (177). Whether increased concentration of butyrate, or other SCFAs due to consumption of rye bread can be observed in peripheral blood in humans is still unclear.

2.4 SUMMARY OF THE REVIEW OF THE LITERATURE

The definition of wholegrain stipulate that it contains the endosperm, germ, and bran in the same relative proportions as in the intact kernel. Wholegrains can be present in a product intact, ground, cracked, or flaked. However, there is no unambiguous definition for wholegrain foods (that is, of how much of wholegrain ingredients a product should contain to be called a wholegrain product). Nevertheless, consumption of wholegrain foods is universally recommended, and approximately three servings of wholegrain foods have been shown to decrease the risk of type 2 diabetes. Despite the recommendations and the evidence from prospective cohort studies, consumption of wholegrains is low in the USA and UK, but in Scandinavian countries it is close to that recommended in the USA. However, in prospective cohort studies wholegrain foods are vaguely described, and it seems that the protective effect of wholegrains is mediated by grain fibre rather than by wholegrain foods per se.

Wholegrain foods, especially of wheat and rye, are a good source of grain fibre. Grain forms a complex structure with different parts and layers which include a mixture of fibre components and bioactive compounds. This natural fibre complex may have synergistic characteristics which are different from those of the isolated fibre fractions. In addition, the structure of cereal foods is important for digestibility and hence probably also for health effects.

Disturbances in glucose metabolism predispose human beings to the development of type 2 diabetes. Significant components in disturbances in glucose metabolism are insulin resistance/decreased insulin sensitivity and impaired first-phase insulin secretion. Intervention studies have produced inconsistent results regarding whether wholegrain foods or foods rich in grain fibre are superior to refined grain foods in improving insulin sensitivity and first-phase insulin secretion. The inconsistencies may be partly due to different methods used to measure insulin sensitivity and first-phase insulin secretion and partly to varying characteristics of the subjects in these studies.

Postprandial studies show that intake of rye bread reduces postprandial insulin response while the form of the postprandial glucose curve is more beneficial as compared to that of white wheat bread. Intake of conventional wholemeal wheat bread does not improve postprandial glucose and/or insulin responses, but an improvement can be achieved when wholemeal wheat bread or white wheat bread is baked with sourdough fermentation.
The protective mechanisms of wholegrain foods or foods rich in grain fibre against type 2 diabetes are not known. They might relate to intestinal effects such as acute postprandial responses and phenomena mediated via the fibre content of cereal foods (Figure 2.2). Grain fibre is fermented in the large intestine, where it may affect the composition of the intestinal microbiota. Intestinal microbiota composition might, in turn, be associated with glucose metabolism, based on theories from animal studies which show that increased absorption of bacterial LPS into the circulation induces low-grade inflammation and insulin resistance. In addition, individuals with type 2 diabetes have differences in their microbiota composition as compared to those without type 2 diabetes. Grain fibre also includes phenolic acids, the absorption of which is generally very low in the small intestine. However, phenolic acids are released and metabolized by the intestinal microbiota. These metabolites might have peripheral effects after absorption into circulation. Fermentation of grain fibre also produces SCFAs, which may reach peripheral circulation in measurable amounts in humans and affect glucose metabolism.

Figure 2.2. Potential effects of wholegrain foods and grain fibre in relation to glucose metabolism.
3 Aims of the Study

This study aimed to investigate potential short- and long-term intestinal effects of wheat and rye foods rich in wholegrains or grain fibre, especially breads, in relation to glucose metabolism in healthy subjects and in subjects with the metabolic syndrome. Postprandial glucose and insulin responses and phenolic acid absorption were measured after consumption of high-fibre breads baked with different technologies, and glucose metabolism and events mediated via the large intestine in intervention settings.

The specific research questions were, whether

1) postprandial glucose and insulin responses to wholemeal wheat bread depend on baking methods (Study I),

2) postprandial glucose and insulin responses are improved after intake of high-fibre white wheat bread enriched with bioprocessed rye bran, and whether absorption of phenolic acids is affected (Study IV),

3) consumption of wholegrain and high-fibre wheat and rye foods affects glucose metabolism in intervention setting (Study II),

4) consumption of wholegrain and high-fibre rye breads versus white wheat bread affects intestinal microbiota composition (Study III),

5) consumption of high-fibre white wheat bread enriched with bioprocessed rye bran affects glucose metabolism similarly to wholegrain rye bread in intervention setting, and whether consumption of rye bread or rye bran is reflected in plasma concentrations of short chain fatty acids (Study V)
4 General Experimental Procedures

4.1 POSTPRANDIAL STUDIES

4.1.1 Subjects
For Studies I and IV, healthy men and women were recruited through advertisements in local newspapers. In Study I, the eleven subjects (seven men, four women) were aged 60 ± 5 y, had a BMI of 32.6 ± 2.9 kg/m² and suffered from the metabolic syndrome. In Study IV, the fifteen subjects (six men, nine women) were aged 57 ± 9 y, had a BMI of 26.1 ± 3.5 kg/m² and varied values for blood glucose, lipid, and blood pressure showing a mean of 5.8 ± 0.5 mmol/l for fasting glucose, 1.4 ± 0.3 mmol/l for HDL cholesterol, 1.4 ± 1.0 mmol/l for triglycerides, and 128 ± 24 / 78 ± 12 mmHg for systolic and diastolic blood pressure. The above values of biochemical measurements were not regarded as inclusion criteria but instead self-reported gastro-intestinal symptoms after consuming cereal foods, especially rye bread. However, this dissertation work does not examine gastrointestinal symptoms.

The subjects were instructed to maintain their living habits, including dietary habits and physical activity, throughout the studies. The weight of the subjects remained unchanged during both postprandial studies, as aimed. Furthermore, the diet of the subjects based on one-day food records remained the same over the day preceding the tests.

4.1.2 Test meals
In Study I, the test breads the subjects ingested were different wheat breads: 1) wholemeal wheat bread, 2) wholemeal wheat bread made with xylanase enzyme treatment, 3) wholemeal wheat bread made using sourdough fermentation, and 4) white wheat bread as a control (Table 4.1).

In Study IV, the three test breads contained rye: 1) wholegrain rye bread, 2) white wheat bread enriched with native rye bran, and 3) white wheat bread enriched with bioprocessed rye bran. White wheat bread was used as a control.

The test breads were baked at VTT Technical Research Centre, Espoo, Finland, except for the commercial wholegrain rye bread in Study IV. The breads were stored in a freezer until they were thawed for serving to the subjects. All the test bread portions contained 50 g available carbohydrate. In Study I, the test meal also included 40 g cucumber and 0.3 l non-caloric berry drink, and in Study IV, 40 g cucumber, 20 g milk-free margarine, and 3 dl water or 1.75 dl coffee/tea and 1.25 dl water. The test meals were served in random order at least three days (Study IV) or one week (Study I) in between every two occasions.

Subjects arrived for the study visits on the morning of the test after a 12-hour fasting period. Their body weight was measured and an intravenous catheter was inserted in the antecubital vein of the arm. A fasting blood sample was taken, and the subjects started to eat the test meal. Meal eating time was restricted to ten (Study I) or fifteen (Study IV) minutes. In Study I, eight postprandial blood samples were taken (at 15, 30, 45, 60, 90, 120,
180, and 240 min); Study IV had five postprandial blood samples (at 30, 60, 120, 180, and 240 min). Furthermore, in Study IV, urine samples were collected for twelve hours before the test meal (basal sample), and at 0-4 h, 4-12 h, and 12-24 h intervals after the test meal. In Study IV, a standardized low-polyphenol lunch including a small portion of the same test bread as eaten in the morning was served to the subjects after the last blood sample was taken. In addition, the standardized lunch included the following food items for optional intake: chicken soup, lettuce, tomato, carrot, salad dressing, margarine, lactose-free milk and sour milk, non-caloric juice, and water. For the rest of the day the subjects were allowed to follow their eating habits.

4.1.3 Glucose and insulin responses
Plasma glucose and plasma and serum insulin were analysed at the Department of Clinical Nutrition by using the glucose dehydrogenase photometric method in Study I and the glucose hexokinase method in Study IV* and KoneLab 20XTi Clinical Chemistry Analyser (Thermo Electron Corp., Vantaa, Finland) for plasma glucose, and the chemiluminescent immunoassay method (Advia Centaur Immunoassay System, Siemens Medical Solution Diagnostics, Tarrytown, NY, USA) for plasma and serum insulin.

In Study I, the maximum increase in glucose and insulin concentrations was calculated by subtracting the highest values of each from the corresponding fasting value. In Study IV, the GP was calculated to describe the form of the glucose curve as follows: time (min) during which the blood glucose concentration was above the fasting concentration was divided by the maximum increase in glucose (100). In both studies, glucose and insulin incremental AUCs were calculated from the AUC above the fasting level including only the area before the concentration dropped below the fasting level by using GraphPad Prism 4.0 for WINDOWS (GraphPad Software, Inc., San Diego, CA).

4.1.4 Phenolic acids
Urine samples collected in Study IV were stored in a freezer at - 80 °C at the Department of Clinical Nutrition. The samples were analysed at VTT Technical Research Centre, Espoo, Finland, for phenolic acids and their metabolites. The methods and reagents used for the analysis are described in detail in Chapter 8. It is noteworthy that the absorbed phenolic acids and their metabolites conjugated in either epithelial cells or the liver were hydrolysed with Helix pomatia enzyme mixture (Sigma G071-500 KU) to be able to report the acids as a sum of non-conjugated and originally glucuronidated phenolic acids.

The excreted phenolic acids (FA, sinapic acid, p-coumaric acid) were reported as such. To clarify reporting the excretion of the twelve individual phenolic acid metabolites analysed, they were grouped into three sets which included the corresponding benzoic, phenylpropionic, and phenylacetic acid metabolites.

*The method in the original study IV on page 86 is incorrect
4.1.5 Statistical analyses
In Study I, the normality of all the variables was tested before the actual analyses using the Kolmogorov-Smirnov test. If not normally distributed, the variables were logarithmic-transformed and the normality was tested again. If the distribution was not normal still, non-parametric tests were chosen.

Overall differences (that is, over the postprandial test among the test breads) in glucose and insulin responses and differences at individual time points between the breads in glucose and insulin concentrations were tested using the General linear model (GLM) for repeated measures. Pairwise comparisons between the breads were adjusted for multiple comparisons (using Bonferroni correction). For non-normally distributed variables, non-parametric Friedman’s test followed by Wilcoxon’s test (for pairwise comparison) was used with adjustment for multiple comparisons.

In Study IV, the overall differences in glucose and insulin responses and differences at individual time points between the breads were tested by the Linear mixed model. Pairwise comparisons were adjusted for multiple comparisons (Bonferroni). The normality of variables was tested within the Linear mixed model using histograms for model residuals. The linear mixed model was also used for comparing differences among and between the test meals in the excretion of phenolic acids and metabolites. For other variables, GLM was used for repeated measures and Friedman’s test followed by Wilcoxon’s test for adjustments for multiple comparisons (Bonferroni).

In both studies, p-values < 0.05 were regarded statistically significant.

4.2 INTERVENTION STUDIES

4.2.1 Subjects
The HealthGrain intervention in Study II was conducted in collaboration with a research group at the University of Naples and included subjects from Kuopio, Finland (n=69; 35 men, 34 women completing the study) and Naples, Italy (n=54; 23 men, 31 women completing the study), aged 60 ± 6 y, with a BMI of 31 ± 4 kg/m² and metabolic syndrome. The inclusion (i.e. metabolic syndrome) and exclusion criteria were similar in both study centres, with the exception that subjects with cholesterol-lowering medication were excluded in Naples and included in Kuopio. Study III was a substudy of the HealthGrain intervention and included only the subjects from Kuopio, Finland (n=51; 25 men, 26 women) who had faecal samples collected at the beginning and at the end of the intervention, with the exclusion of one subject with inflammatory bowel disease.

Study V included healthy subjects from Kuopio (n=21; 9 women, 12 men completing the intervention) who were recruited based on self-reported gastrointestinal symptoms after consuming cereal foods, especially rye bread. The baseline characteristics of the subjects were: age 56 ± 7 y, BMI 25 ± 2.8 kg/m², waist circumference 92 ± 7 cm (men) / 79 ± 7 cm (women), fasting glucose 5.4 ± 0.4 mmol/l, systolic and diastolic blood pressure 137 ± 20 / 85
± 7 mmHg, HDL-cholesterol 1.3 ± 0.2 mmol/l (men) / 1.9 ± 0.4 mmol/l (women), and triglycerides 0.9 ± 0.4 mmol/l.

4.2.2 Diets
In Study II, the subjects in the intervention study arm consumed wholegrain and fibre-rich grain foods while subjects in the control group consumed refined grain foods for twelve weeks. The test diets in the two study centres differed slightly because of cultural differences. In Naples, the wholegrain intervention diet included sourdough-fermented wholemeal wheat bread (90% of the bread consumed) and Finnish endosperm rye bread (10% of the bread consumed), wholemeal wheat pasta, barley kernels, oat biscuits (Eloven, Raisio), and bran breakfast cereals (Table 4.1). The control diet included white wheat bread, rice, cornmeal porridge, rice breakfast cereals, and pizza made of white wheat flour. In Kuopio, the intervention diet rich in grain fibre included wholegrain rye bread (50% of the breads consumed), endosperm rye bread (40% of the breads consumed), and sourdough-fermented wholemeal wheat bread (10% of the breads consumed) (which was the same bread as in Study I), wholemeal wheat pasta, and oat biscuits (Eloven, Raisio) for voluntary intake. The control diet included white wheat breads, and only 1-2 small portions of rye products were allowed daily. Thus, both the intervention and control diets in Naples and Kuopio were based on wholegrain and/or high-fibre grain foods and refined grain foods, respectively, and the only difference between the study centres was that in Naples, the intervention diet was based on wheat, whereas in Kuopio it was based on rye.

In Study III, the subjects consumed the test products provided at the Kuopio study centre in the HealthGrain intervention. In Study V, the subjects consumed white wheat bread during the four-week run-in period, and wholegrain rye bread and white wheat bread enriched with bioprocessed rye bran in cross-over manner during the following two four-week periods.

In both intervention studies, the subjects were instructed to maintain their living habits, including dietary habits and physical activity, throughout the studies. As aimed, the background diets of the subjects based on four-day (Kuopio, Finland) or seven-day (Naples, Italy) food records remained the same over the interventions, except for changes in nutrient intakes which were explained by the consumption of the wholegrain and fibre-rich and refined grain test products. In Study II, daily energy intake increased during the intervention in the intervention group by 200 kcal and in the control group by 250 kcal, but the weight of the subjects remained the same as in Study V.

4.2.3 Measurement of glucose metabolism
In both the intervention studies, blood samples were taken for measuring fasting concentrations of glucose and insulin at the beginning and at the end of the intervention. Furthermore, in Study II, insulin sensitivity was analysed intravenously by the FSIGT as following: a glucose dose of 300 mg/kg body weight was given intravenously. After 20 min, a bolus of 0.03 U/kg of insulin was injected. Blood samples were collected frequently within a period of three hours for the measurement of glucose and insulin concentrations. The glucose and insulin concentration were used to calculate the insulin sensitivity index (SI) using the minimal model (MINMOD) computer program (180). Insulin sensitivity was also
analysed at the fasting state using QUICKI (1/\(\ln(\text{insulin 0 min}) + \ln(\text{glucose 0 min})\)). First-phase insulin secretion was evaluated from the FSIGT using mean insulin concentration (from 2 to 10 min) and expressed as \(\Delta\text{AIRc}\) (mU/ml). DI, which takes insulin resistance into account when measuring the ability of pancreatic beta cells to increase insulin secretion, was calculated as \(\text{Si} \times \Delta\text{AIRc}\) (78).

In Study V, a three-hour standardized meal test was performed at the end of each test bread period to evaluate postprandial glucose metabolism. Blood samples were taken at 0, 30, 60, 120, and 180 min at fasting and after subjects started eating the test meal, and all samples excluding the 180-min one were analysed for glucose and insulin responses. Glucose and insulin AUCs were calculated from the 0-120 min AUC above the fasting level. First-phase insulin secretion was evaluated from fasting and 30 min postprandial glucose and insulin concentrations by calculating insulinogenic index (IGI; (insulin 30 min – 0 min) / (glucose 30 min – 0 min)). DI was calculated by multiplying IGI with a measure of insulin sensitivity (ISIcomp \((10000/ \sqrt{(G_0 \times I_0 \times G_M \times I_M)}\) (78) \((G_0 = \text{fasting glucose concentration}; I_0 = \text{fasting insulin concentration}; G_M = \text{mean of postprandial glucose concentrations}; I_M = \text{mean of postprandial insulin concentrations}).

In Kuopio, plasma glucose and serum insulin were analysed using the glucose hexokinase method (Konelab System Reagents; Thermo Fisher Scientific, Vantaa, Finland) and chemiluminescent immunoassay (Advia Centaur, Siemens Medical Solution Diagnostics, Tarrytown, NY, USA), respectively. In Naples, plasma glucose was analysed by enzymatic colourimetric method on a Cobas Mira autoanalyser (ABX Diagnostics, Montpellier, France). Plasma insulin was analysed by an enzyme-linked immunosorbent assay (ELISA) for the specific determination of biologically active insulin (DAKO Insulin, DAKO Diagnostics, Ely, UK).

**4.2.4 Intestinal microbiota and short chain fatty acids**

In Study III, spot faecal samples were collected from the subjects at the beginning and at the end of the HealthGrain intervention. The samples were stored in a freezer at -70 \(^\circ\)C at the Department of Clinical Nutrition until they were sent to the University of Wageningen for microbiota composition analysis. The composition of the intestinal microbiota was analysed using the Human Intestinal Tract Chip (HITChip) (181).

In Study V, blood samples for analysis of SCFAs were collected during the standardized meal test and analysed from the samples collected at the fasting state and at 30 min after commencement of the test meal. The plasma samples were sent to the University of Aarhus for SCFA analysis by gas chromatography (182).

**4.2.5 Statistical analyses**

In Study II, GLM for univariate analysis was used to test differences between the groups in relative changes (calculated as change from baseline) of insulin sensitivity and first-phase insulin secretion; GLM for repeated measures was used to test differences between the groups in changes of dietary and anthropometric variables, with the study centre and baseline variables as covariates. A paired samples t-test was used to test intra-group
changes in the variables. The normality of the variables was tested using the Shapiro-Wilk’s test, and the variables were normalized with logarithmic transformation where necessary.

In Study III, a biostatistician from the University of Helsinki was responsible for performing statistical analyses which are described in detail in the Chapter 7. In short, logarithmic-transformed variables were used for all the analyses. The linear mixed model was used to compare differences between groups and the paired-samples t-test to compare differences within a group. P-values were corrected for multiple comparisons with the Benjamini-Hochberg false discovery rate (FDR).

In Study V, GLM for repeated measures was used for normally distributed variables (with or without preceding logarithmic transformation) and Friedman’s test followed by Wilcoxon’s test for non-normally distributed variables. Because of the experimental design of the meal test, p-values for markers of glucose metabolism were not adjusted for multiple comparisons.

In all the studies, p-values < 0.05 were regarded statistically significant.
<table>
<thead>
<tr>
<th>Study</th>
<th>Test products (g)</th>
<th>Consumed amount (g)</th>
<th>Grain fibre intake (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>wholemeal wheat bread</td>
<td>129</td>
<td>8.4</td>
</tr>
<tr>
<td></td>
<td>wholemeal wheat bread baked with xylanase enzyme treatment</td>
<td>129</td>
<td>8.5</td>
</tr>
<tr>
<td></td>
<td>sourdough wholemeal wheat bread</td>
<td>134</td>
<td>8.6</td>
</tr>
<tr>
<td></td>
<td>white wheat bread</td>
<td>109</td>
<td>3.3</td>
</tr>
<tr>
<td>II</td>
<td>Kuopio study centre: sourdough wholegrain rye bread</td>
<td>87 ± 26</td>
<td>24 ± 1 from the wholegrain and high-fibre test products, 10 ± 0 from the control products</td>
</tr>
<tr>
<td></td>
<td>sourdough endosperm rye bread</td>
<td>73 ± 21</td>
<td></td>
</tr>
<tr>
<td></td>
<td>sourdough wholemeal wheat bread</td>
<td>26 ± 4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>wholemeal wheat pasta</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td></td>
<td>oat biscuits</td>
<td>13 ± 15</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Naples study centre: sourdough wholemeal wheat bread</td>
<td>139 ± 25</td>
<td></td>
</tr>
<tr>
<td></td>
<td>sourdough endosperm rye bread</td>
<td>14 ± 4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>wholemeal wheat pasta</td>
<td>86 ± 23</td>
<td></td>
</tr>
<tr>
<td></td>
<td>barley kernels</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td></td>
<td>oat biscuits</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td></td>
<td>bran breakfast cereals</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td></td>
<td>control diets: white wheat breads and other refined cereal foods, and restriction of rye products</td>
<td>197 ± 46 and 136 ± 47 of white wheat breads in Kuopio and Naples, respectively; in Naples 78 ± 19 of refined wheat pasta, rice, and pizza</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>sourdough wholegrain rye bread</td>
<td>92 (sourdough wholegrain rye and wholemeal wheat breads)</td>
<td>19 from the wholegrain and high-fibre test products, 10 from the control products</td>
</tr>
<tr>
<td></td>
<td>sourdough wholemeal wheat bread</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td></td>
<td>sourdough endosperm rye bread</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td></td>
<td>wholemeal wheat pasta</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>control diet: white wheat breads and restriction of rye products</td>
<td>188 of white wheat breads</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>sourdough wholegrain rye bread</td>
<td>123$^2$</td>
<td>16.4$^2$</td>
</tr>
<tr>
<td></td>
<td>white wheat bread enriched with native rye bran</td>
<td>164$^2$</td>
<td>19.1$^2$</td>
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<tr>
<td></td>
<td>white wheat bread enriched with bioprocessed rye bran</td>
<td>166$^2$</td>
<td>16.8$^2$</td>
</tr>
<tr>
<td></td>
<td>white wheat bread</td>
<td>109$^2$</td>
<td>3.8$^2$</td>
</tr>
<tr>
<td>V</td>
<td>sourdough wholegrain rye bread</td>
<td>205 ± 50</td>
<td>21 ± 5</td>
</tr>
<tr>
<td></td>
<td>white wheat bread enriched with bioprocessed rye bran</td>
<td>195 ± 53</td>
<td>20 ± 5</td>
</tr>
<tr>
<td></td>
<td>white wheat bread</td>
<td>169± 24</td>
<td>5 ± 1</td>
</tr>
</tbody>
</table>

$^1$ per portion (postprandial studies) or per day (intervention studies); $^2$ at breakfast; ND, no data
5 Sourdough fermentation of wholemeal wheat bread increases solubility of arabinoxylan and protein and decreases postprandial glucose and insulin responses\(^1\) (Study I)

### 5.1 ABSTRACT

Glycemic responses to most of the conventional breads are high, including breads made of wholemeal flour. Baking technology is known to affect these responses. The aim of the present study was to investigate effects of xylanase enzyme treatment and sourdough fermentation in wholemeal wheat bread baking on postprandial glucose and insulin responses and on *in vitro* protein digestibility. The wheat breads were made of 100 % flour from peeled kernels by straight dough or sourdough fermentation method, and with or without using of xylanase during mixing of dough. Standard white wheat bread was used as reference. All test bread portions contained 50 g available carbohydrate and were served in random order to eleven insulin resistant subjects. Blood samples for measuring glucose and insulin concentrations were drawn over four hours. The sourdough wholemeal wheat bread resulted in the lowest postprandial glucose and insulin responses among the four tested breads (treatment x time; \(p=0.000\) and \(p=0.022\), respectively). There were differences in solubility and depolymerisation of protein and arabinoxylan among the breads but these did not fully explain the *in vivo* findings. In conclusion, the health effects of wholemeal wheat bread can be further improved by using sourdough process in breadmaking.

### 5.2 INTRODUCTION

Whole grain foods as a part of the diet have many beneficial health effects in humans. Recent meta-analyses show that increased consumption of whole grain foods decreases the risk of type 2 diabetes (17) and cardiovascular diseases (18). Diets with low glycemic index (GI) and/or glycemic load have similar protective effects (183). Basically, consumption of high GI foods leads to repeated high postprandial glucose and insulin concentrations, which may contribute to the development of insulin resistance and the incidence of type 2 diabetes (97). Diets low in GI and high in indigestible carbohydrates have beneficial effects on diabetes risk factors in overweight persons with impaired glucose tolerance (184).

Whole grains contain the starchy endosperm, germ and bran of the grain, which are present in the same relative proportions as they exist in the intact caryopsis (9). The components in whole grains that are assumed to have beneficial health effects include amount and type of

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fibre and presence of bioactive compounds which are concentrated in the bran layers of the grain (185). When grains are refined by removing the bran, the content of most of these compounds is decreased.

Wheat is the most consumed grain globally (186) and it is commonly used as bread in many households. Unfortunately, glycemic responses to most of the conventional wheat breads are known to be high, also to breads that are made of wholemeal flour (187). To our knowledge, there are no breads on the market that are made of 100 % wholemeal wheat flour and have low glycemic responses. On the contrary, rye bread is used only in Northern and Eastern Europe, despite being rich in many nutrients and bioactive compounds and newly reported beneficial effects on insulin metabolism. In our previous studies, sourdough fermented rye bread was shown to produce beneficial effects on postprandial insulin responses as compared to white wheat bread or wholemeal wheat and oat breads, both in healthy persons (62) and in persons with the metabolic syndrome (188). These studies suggest that reduced postprandial insulin response after rye bread intake may result from bread structure. Furthermore, increasing the content of soluble arabinoxylan in bread improves postprandial glucose and insulin responses in a dose dependent manner (189).

The aim in the present study was to investigate effects of sourdough fermentation and xylanase enzyme treatment in wholemeal wheat bread baking on postprandial glucose and insulin responses. To explore mechanisms underlying the postprandial responses, effects of baking technology on the state of protein and arabinoxylan in bread were analysed. The test breads were baked of flour from peeled kernels, because peeling of grains provides technological advances both in the palatability and safety of the products.

5.3 EXPERIMENTAL

5.3.1 Test breads
Four different wheat breads were produced. White wheat bread, wholemeal wheat (WM) bread and WM bread made with xylanase were prepared with straight dough method. WM bread was also made using a sourdough fermentation method. For the white wheat bread, standard flour T550 of variety Tiger (Bühler, Uzwil, Switzerland) was used as a flour material. The WM breads were made of 100 % wheat flour from peeled (3.5 % removal of outermost layers) grains (variety Tiger, Bühler, Uzwil, Switzerland). Xylanase from Bacillus subtilis (from Danisco) was dosed as 5 nkat /g flour, based on activity assayed as described by Bailey et al. (190). Sourdough was prepared by mixing the starter culture LA4 (0.4 % of dough weight (d.w.)) (Lallemand, France) and WM flour (37.4 % d.w.) with water. The sourdough was fermented for 19.5 h at 30°C to obtain dough pH value of 3.9-4.1 and total titratable acidity value 16-17. The baked bread loaves were frozen and stored in a freezer at -18 °C until they were thawed in fridge overnight before every study visit. Before serving to the subjects, the breads were sliced, preserving the crust.

5.3.2 Chemical analyses of the breads
Content of protein (by Kjeldahl method; (191)), total dietary fibre (by Enzymatic-Gravimetric method; (192)), fat (by Fat in Flour–Mojonnier method; (193)), arabinoxylans
(AX) (194), and digestible starch (i.e. available carbohydrate) (Megazyme method; (195)) were determined from the test breads. Moisture content was determined using a two-phase moisture measurement; pre-drying of samples at 40 °C and then at 130 °C. Acidity of breads was determined by measuring the pH value and total titratable acidity ((196). The effect of peeling process used in the present study on preservation of the potentially bioactive compounds characteristic of whole grains was studied by determining the content of phenolic acids. Ferulic acid, sinapic acid and p-coumaric acid) were analysed using a method described by Mattila et al. (66). All the analyses were made in duplicate.

5.3.3 In vitro digestibility of protein of the breads

Hydrolysis of bread samples with alpha-amylase and pepsin

In vitro hydrolysis of protein of the test breads was performed in duplicate using a sequential amylase and pepsin treatment to mimic hydrolysis reactions in the mouth and stomach, respectively. 100 mg of freeze-dried milled bread sample was pre-hydrated for 15 min at 40 °C in 0.5 ml 0.2 M acetate buffer, pH 5. Amylase (fungal source, Megazyme; dosage: 2.5 U / g freeze-dried bread sample) was added to the pre-hydrated suspension. After the incubation with amylase for 10 min, suspension pH was adjusted to 2.0, after which pepsin (2000 FIP U/g, Merck; dosage: 0.4 FIP U/g freeze-dried bread sample) was added. Samples were collected after pepsin incubation times of 0, 5, 15, 30, 60, 90, 120 and 150 min. Suspension pH was adjusted to 5.0 to terminate the enzymatic reaction, after which centrifugation of samples was performed to remove the solids. Content of solubilised protein in the supernatant was measured by Bio-Rad DC protein assay kit (Bio-Rad, Richmond, CA, USA) using bovine serum albumin as standard. To monitor the molecular weight (MW) range of the protein hydrolysates of different time points, SDS PAGE was performed with ready-made 12 % Tris-HCl Ready Gels (Bio-Rad) as described by Laemmli (197). Samples were loaded to the gels in volume based (45µl). Pre-stained standards (Bio-Rad 161-0318), with a MW range 194.7, 116.5, 97.2, 50.2, 37.6, 29.3, 20.0, 7.2 kDa, were used as proteins standards.

Extraction of proteins of the breads (without pepsin treatment)

To analyse the content of soluble proteins in the untreated breads, extraction of sodium dodecyl sulphate (SDS) -soluble and DTT (dithiothreitol) -soluble proteins were performed with a modified method described by Primo-Martín (198). Freeze-dried bread samples were extracted with constant mixing in 1.5% SDS for 30 min at 20 °C and centrifuged. For further extraction of DTT-soluble proteins, remaining solids were extracted (30 min) with 2 % DTT (and 1.5 % SDS) and centrifuged. The supernatants were boiled for 10 min, centrifuged, and analysed by SDS PAGE.

5.3.4 Molecular weight distribution of water-extractable arabinoxylans of the breads

Analysis of the MW distribution of water-extractable AX was performed by HPLC analysis, modified from the HPLC method described by Suortti (199). For sample preparation, freeze-dried bread samples (200 mg) were solubilised in 5 ml of 50 mM Na-phosphate buffer (pH 4.5), boiled for 30 minutes and centrifuged. To remove other polysaccharides than AX, α- and β-dextrins present in supernatant were hydrolysed by Optidex L-400
(Genencor International, Ltd) at 60 °C for 4 h. After incubation the samples were boiled for 30 minutes and analysed by HPLC using M-2414 refractive index detector.

5.3.5 Subjects
Thirteen Finnish adults (age 40-65 y) were recruited for the study. The criteria for the entry to the study were impaired fasting glycaemia (IFG) (fasting plasma glucose concentration 5.6-6.9 mmol/l), BMI 26-39 kg/m², and at least two other features of the metabolic syndrome according to the Adult Treatment Panel III criteria (200) as modified by the AHA (77): waist circumference >102 cm (males)/ >88 cm (females), fasting serum triacylglycerol concentration >1.7 mmol/l, fasting serum HDL-cholesterol concentration <1.0 mmol/l (males)/ <1.3 mmol/l (females), blood pressure ≥ 130/ ≥ 85 mmHg, or medication for hypertension. Exclusion criteria were BMI ≥ 40 kg/m², serum triacylglycerol concentration >3.5 mmol/l, serum total cholesterol concentration >8 mmol/l, type 1 or 2 diabetes, abnormal liver, thyroid, or renal function, alcohol abuse (>16 portions/week (females)/ >24 portions/week (males)) and inflammatory intestinal disease.

One man and one woman dropped out of the study because of personal reasons. The final number of the subjects was eleven. The protocol for the study was approved by the Ethics Committee of the Hospital District of Northern Savo.

5.3.6 Postprandial study
Subjects arrived for the study visits on the test morning after 12-hour fasting. Their body weight was measured and an intravenous catheter was inserted in the antecubital vein of the arm. A fasting blood sample was taken, and the subjects started to eat the test meal. The test meals were served repeatedly in random order one week between each occasion, with each subject acting as his/her own control. The test portion contained 50 g available carbohydrate from one of the sliced test breads with crust, and 40 g of cucumber and 0.3 l of non-caloric berry drink (Fun Light, Felix Abba, Kumla, Sweden) to ease the eating of the bread. Meal eating time was restricted to ten minutes, and the eating time for every subject was documented. Eight blood samples were taken after the start of eating (15, 30, 45, 60, 90, 120, 180, and 240 min) for the measurement of plasma glucose, and serum insulin and C-peptide.

Known confounding factors were standardised by advising subjects to maintain their body weight and habitual living habits throughout the study. Heavy exercise was forbidden on the day before each test, as well as smoking on the test morning. The subjects were asked to arrive for the study visits by car or bus, if possible, to avoid extra physical activity. Furthermore, they were advised to maintain their habitual diet, avoid unusually large portions of food on the day preceding each test, and avoid consumption of alcohol for 2 days before each study visit. Nutrient intakes based on one-day food record preceding every study visit were calculated by using the Micro-Nutrica 2.5 Software (Finnish Social Insurance Institution, Turku, Finland).

5.3.7 Biochemical analyses
Plasma glucose was analysed by using the glucose dehydrogenase photometric method (Konelab, Thermo Electron Corp., Vantaa, Finland) and KoneLab 20XTi Clinical Chemistry Analyser (Thermo Electron Corp., Vantaa, Finland). Serum insulin and C-peptide were
analysed by the chemiluminescent immunoassay method (Advia Centaur, Bayer, USA; Siemens Medical Solution Diagnostics, Tarrytown, NY).

Maximum increase in glucose, insulin, and C-peptide concentrations were calculated by subtracting the highest value of each from the corresponding fasting value. Glucose, insulin, and C-peptide areas under the curve (AUC) were calculated from the area beneath the curve above the fasting level including only the area before the concentration dropped below the fasting level by using GraphPad Prism 4.0 for WINDOWS (GraphPad Software, Inc., San Diego, CA).

5.3.8 Statistical analyses
Overall differences in postprandial glucose, insulin, and C-peptide concentrations among the four tested breads were analysed using General Linear Model (GLM) for repeated measures (SPSS for WINDOWS, SPSS Inc. v. 14.0, Chicago, IL). For pairwise comparisons GLM was followed by a Post Hoc test (Bonferroni). The normality of variables for GLM was tested by Kolmogorov-Smirnov test with Lilliefors correction.

For glucose, insulin, and C-peptide AUC and maximum increase, eating times, and subjects' weight and diet the statistical significance was assessed by the nonparametric Friedman’s test followed by the Wilcoxon’s test with the Bonferroni correction for multiple comparisons. Analyses, in which p-values were < 0.05, were considered as statistically significant. Statistical data are expressed as mean ± S.D.

5.4 RESULTS

5.4.1 Characteristics of the test breads
The proportions of fibre and fat were lower in the white wheat bread portion compared with the WM bread portions (Table 5.1). The sourdough and enzyme treatments did not yield differences in the chemical composition of WM breads, except the increased content of water-extractable AX by xylanase addition (Table 5.1). The pH value of the white wheat, WM and xylanase WM bread was around 6, but sourdough process decreased the pH to 4.6 (Table 5.1). The amount of total phenolic acids was clearly higher in the WM breads than in the white wheat bread, and not different between the WM breads (Table 5.1).
Table 5.1. Nutrient composition and pH of the test bread portions

<table>
<thead>
<tr>
<th></th>
<th>White wheat bread</th>
<th>WM¹ bread</th>
<th>Xylanase WM¹ bread</th>
<th>Sourdough WM¹ bread</th>
</tr>
</thead>
<tbody>
<tr>
<td>Portion size (g)</td>
<td>109</td>
<td>129</td>
<td>129</td>
<td>134</td>
</tr>
<tr>
<td>Energy (kcal)</td>
<td>253</td>
<td>271</td>
<td>270</td>
<td>276</td>
</tr>
<tr>
<td>Available carbohydrate (g)</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Protein (g)</td>
<td>9.9</td>
<td>12.0</td>
<td>12.0</td>
<td>12.5</td>
</tr>
<tr>
<td>Fat (g)</td>
<td>1.5</td>
<td>2.6</td>
<td>2.5</td>
<td>2.8</td>
</tr>
<tr>
<td>Total dietary fiber (g)</td>
<td>3.3</td>
<td>8.4</td>
<td>8.5</td>
<td>8.6</td>
</tr>
<tr>
<td>Total arabinoyxylan (g)</td>
<td>2.0</td>
<td>5.5</td>
<td>5.2</td>
<td>5.9</td>
</tr>
<tr>
<td>Water-extractable</td>
<td>0.6</td>
<td>0.7</td>
<td>1.9</td>
<td>0.9</td>
</tr>
<tr>
<td>arabinoyxylan (g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Moisture (g)</td>
<td>42.0</td>
<td>53.1</td>
<td>52.6</td>
<td>55.6</td>
</tr>
<tr>
<td>Phenolic acids²</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ferulic acid</td>
<td>110</td>
<td>744</td>
<td>721</td>
<td>725</td>
</tr>
<tr>
<td>sinapic acid</td>
<td>11</td>
<td>64</td>
<td>64</td>
<td>58</td>
</tr>
<tr>
<td>p-coumaric acid</td>
<td>4</td>
<td>23</td>
<td>22</td>
<td>23</td>
</tr>
<tr>
<td>pH</td>
<td>5.9</td>
<td>6.0</td>
<td>6.0</td>
<td>4.6</td>
</tr>
<tr>
<td>Titratable acidity³</td>
<td>2.3</td>
<td>3.8</td>
<td>3.8</td>
<td>11.5</td>
</tr>
</tbody>
</table>

¹WM=wholemeal wheat
²µg/g of dry matter of bread: total amount, calculated from free and esterified amounts
³ml 0.1 M NaOH used to titrate 10 g bread

5.4.2 In vitro digestibility of protein of the breads
In the untreated breads (i.e. the starting point), the soluble protein content was highest in the sourdough WM bread, whereas the xylanase, WM, and white wheat bread had less solubilised proteins (Fig. 5.1). SDS PAGE analysis of the bread proteins revealed that more solubilised and smaller MW proteins and peptides were present in the bread after the sourdough process (Fig. 5.2). However, the protein hydrolysis rate by pepsin treatment in vitro was similar among the breads (Fig. 5.1).
**Fig. 5.1.** In vitro digestion of protein of the test breads by amylase and pepsin, followed as a function of incubation time, calculated based on the amount (%) of soluble protein of bread (dry matter). Time frame: -15 to 0 min, amylase treatment; 0 to 185 min, pepsin treatment.

**Fig. 5.2.** In vitro digestion of proteins of the WM and sourdough WM test breads by pepsin, followed as a function of incubation time. Analysis of protein hydrolysate by SDS PAGE. Lanes: MW, molecular weight marker; 1. SDS extract (no pepsin); 2. DTT extract (no pepsin); 3. -10 min (pre-hydration); 4. 5 min (pepsin); 5. 15 min (pepsin); 6. 30 min (pepsin); 7. 60 min (pepsin); 8. 90 min (pepsin); 9. 120 min (pepsin); 10. 150 min (pepsin). WM=wholemeal wheat
5.4.3 Molecular weight distribution of water-extractable arabinoxylans of the breads

In the xylanase WM bread, the MW of water-extractable AX was clearly smaller than in other breads (Fig. 5.3). Xylanase caused decrease of MW from 540-120 kDa to 100-10 kDa. On the other hand, the amount of high MW water-extractable AX (540-120 kDa) was highest in the sourdough WM bread.

![Graph showing molecular weight distribution of water-extractable arabinoxylans of the test breads. Detection using refractive index (RI), 410 mV. Elution of pullulan standards (540 kDa, 112 kDa, 47.3 kDa and 5.8 kDa) shown at X-axis.](image)

Fig. 5.3. Molecular weight distribution of water-extractable arabinoxylans of the test breads. Detection using refractive index (RI), 410 mV. Elution of pullulan standards (540 kDa, 112 kDa, 47.3 kDa and 5.8 kDa) shown at X-axis.

5.4.4 Basic characteristics of the study subjects

The baseline characteristics of the subjects (seven men, four women) were the following: age 60 ± 5.2 y, BMI 32.6 ± 2.9 kg/m², waist circumference 111.3 ± 10.1 cm, diastolic blood pressure 136 ± 19 mmHg, systolic blood pressure 90 ± 11 mmHg, fasting plasma glucose 6.1 ± 0.3 mmol/l, fasting serum cholesterol 5.5 ± 0.6 mmol/l, fasting serum LDL-cholesterol 3.5 ± 0.4 mmol/l, fasting serum HDL cholesterol 1.4 ± 0.2 mmol/l, and fasting serum triglycerides 1.8 ± 0.9 mmol/l. The weights of the subjects remained unchanged throughout the study, as did the mean energy, protein, fat, carbohydrate, starch, fibre, and alcohol intake calculated from the food records (data not shown).

5.4.5 Postprandial plasma glucose, and serum insulin and C-peptide responses

Postprandial glucose, insulin, and C-peptide responses during 240 minutes after the test meal differed significantly among the four breads (treatment-by-time interaction, p=0.000; p=0.022; p=0.003, respectively). Plasma glucose concentration after ingestion of the sourdough WM bread was significantly lower at time points 45, 60, 90 and 120 min (p=0.000 – p=0.017) and higher at 240 min (p=0.006) compared with the white wheat bread (Fig. 5.4
A). In addition, plasma glucose concentration after ingestion of the sourdough WM bread was significantly lower than that of the WM bread at a time point 30 min (p=0.035). Glucose AUC was significantly smaller and maximum increase in glucose significantly lower for the sourdough WM bread than for the white wheat bread (p=0.012 and p=0.036, respectively) after eating of the breads (Table 5.2).

Serum insulin concentration was significantly lower at the time points 0 min (p=0.004) and 90 min (p=0.024) in the sourdough WM bread meal test compared with the white wheat bread meal test (Fig. 5.4 B), and significantly lower at 60 min after eating of the sourdough WM bread compared to the xylanase WM bread (p=0.034). There were no significant differences in insulin AUC between the test breads, but the maximum increase in insulin concentration after the sourdough WM bread was significantly lower than after the white wheat bread (p=0.040) (Table 5.2).

Serum C-peptide concentration after ingestion of the sourdough WM bread was significantly lower only at the time point 120 min compared with that of the white wheat bread (p=0.007) (data not shown). There were no significant postprandial differences in C-peptide AUC among the test breads after adjustment of the Bonferroni correction (Table 5.2). However, the maximum increase in C-peptide concentration was significantly lower after the sourdough WM bread than the white wheat bread (p=0.030).

Times to reach the maximum increase in glucose, insulin, and C-peptide concentrations did not differ among the test breads (data not shown). The meal eating time was the same for each of the test breads: 7.5 ± 1.7, 7.7 ± 1.7, 7.7 ± 1.8 and 7.6 ± 1.8 min for the white wheat bread, WM bread, xylanase WM bread, and sourdough WM bread meals, respectively.

Table 5.2. Maximum increase in glucose, insulin and C-peptide concentrations and areas under the curve in response to the ingestion of the test breads

<table>
<thead>
<tr>
<th>Maximum increase</th>
<th>White wheat bread</th>
<th>WM bread</th>
<th>Xylanase WM bread</th>
<th>Sourdough WM bread</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mmol/l)</td>
<td>3.6 ± 0.9</td>
<td>3.4 ± 1.0</td>
<td>3.4 ± 1.2</td>
<td>2.9 ± 1.0(^b)</td>
</tr>
<tr>
<td>Insulin (mU/l)</td>
<td>91.2 ± 36.2</td>
<td>92.3 ± 40.9</td>
<td>95.3 ± 43.8</td>
<td>78.5 ± 36.2(^b)</td>
</tr>
<tr>
<td>C-peptide (nmol/l)</td>
<td>2.3 ± 0.5</td>
<td>2.1 ± 0.6</td>
<td>2.0 ± 0.8</td>
<td>1.9 ± 0.5(^b)</td>
</tr>
<tr>
<td>Area under the curve</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose (mmol/l x min)</td>
<td>272 ± 134</td>
<td>204 ± 86</td>
<td>212 ± 125</td>
<td>173 ± 105(^b)</td>
</tr>
<tr>
<td>Insulin (mU/l x min)</td>
<td>8651 ± 4097</td>
<td>8064 ± 4222</td>
<td>8423 ± 4807</td>
<td>7194 ± 3797</td>
</tr>
<tr>
<td>C-peptide (nmol/l x min)</td>
<td>279 ± 77</td>
<td>254 ± 87</td>
<td>250 ± 87</td>
<td>232 ± 72</td>
</tr>
</tbody>
</table>

\(^a\) mean ± S.D., n=11  
\(^b\) significantly different from white wheat bread, p<0.05 (Wilcoxon’s test corrected with Bonferroni)
Fig. 5.4. Mean fasting and postprandial A) plasma glucose responses to all breads and B) serum insulin responses to all breads, n=11. White wheat bread (■), WM bread (▲), xylanase WM bread (×) and sourdough WM bread (○). The sourdough WM bread differed significantly from the white wheat bread (aaa p<0.001, aa p<0.01, a p<0.05), from the WM bread (b p<0.05) and from the xylanase WM bread (c p<0.05) (General Linear Model for Repeated Measures with Bonferroni adjustment).

5.5 DISCUSSION

In the present study, the aim was to investigate whether the sourdough fermentation method or addition of xylanase into the dough affects postprandial glucose and insulin responses to wholemeal wheat bread. Furthermore, influence of baking technology on the state of protein and arabininoxylan in bread was analysed to explore mechanisms underlying the postprandial responses. All the test breads made of peeled kernels contained high amounts of dietary fibre and phenolic acids compared to the white wheat bread. The peeling process removes only a small amount of the outermost layers of the bran, and preserves a large amount of the compounds characteristic of whole grains.

Intake of the sourdough WM bread resulted in retarded postprandial glucose and insulin responses compared with the white wheat bread. On the other hand, intake of the xylanase WM bread did not improve the postprandial responses. Also the regular WM bread produced similar postprandial responses to the white wheat bread in the present study, as expected from the results in other postprandial studies on wholemeal wheat breads (102,201,202).
Sourdough fermentation of bread has in the present and previous studies (116,117,203) resulted in decreased postprandial glucose and insulin responses. De Angelis et al. (203) and Liljeberg et al. (117) reported retarded hydrolysis rate of starch from sourdough fermented breads measured in vitro, but Scazzina et al. (116), using a similar in vitro method, did not find any difference in the rate of starch digestion between the breads made by sourdough fermentation and yeast leavening, despite reduced postprandial glucose responses after ingestion of the sourdough fermented bread. In the present study, we did not investigate the in vitro hydrolysis rate of starch in the phase mimicking the pancreatic digestion of starch.

One explanation for the reduced postprandial responses could be delayed gastric emptying rate due to organic acids other than lactic acid produced by sourdough microflora (118,119). However, Najjar et al. (104) did not detect any difference in the gastric emptying rate between white wheat bread and similar bread made by sourdough fermentation. They estimated gastric emptying rate by adding paracetamol in the flour of the breads, and measuring the postprandial concentration in blood (104). The gastric emptying rate in vivo was not measured in the present study. The pH of the sourdough WM bread in the present study was 4.6 which is slightly lower than the pH of 5.07 of the sourdough bread Najjar et al. (104) used. Liljeberg & Björck (118) reported the pH of the bread with added lactic acid to have been 3.9 which had no delaying effect on the gastric emptying rate.

Another possible contributing factor that should be considered in more detail is proteolysis, known to happen in sourdough (204). Although the in vitro analysis revealed no differences in the hydrolysis rate of protein among the breads in the present study, the content and MW of soluble protein varied. The amount of soluble protein was highest and the MW of those proteins lowest in the sourdough WM bread sample compared with samples of the other three breads, indicating that more solubilised protein is ingested with sourdough WM bread when compared to the other breads. Ingestion of carbohydrate solution containing wheat protein hydrolysate has been shown to stimulate insulin secretion more than a similar solution with intact milk protein (205). Consequently, a higher insulin response can be expected after ingestion of the sourdough WM bread in which proteins are degraded by fermentation, as compared to the breads with more intact protein. In contrast to this, the postprandial insulin response to the sourdough WM bread in the present study tended to be lower than to the white wheat bread. However, it is possible that structural factors (solid bread vs. liquid) and protein-starch interaction may affect the results (206). To our knowledge, there are no reported studies testing the effect of wheat protein hydrolysates versus intact wheat protein on postprandial insulin responses.

It is known that soluble fibre with high MW retards postprandial glucose and insulin responses by increasing viscosity (207). Lu et al. (189) reported lowered postprandial glucose and insulin responses after ingestion of white wheat bread containing 6 g added AX-rich fiber. In the present study, the WM test bread portions contained approximately 6 g total AX of which 37 % was water-extractable in the xylanase WM bread as a result of xylanase treatment, whereas the proportion of water-extractable AX was naturally smaller in the other WM breads (13 and 15 % for WM and sourdough WM bread, respectively).
However, the postprandial glucose and insulin responses were not improved after ingestion of the xylanase WM bread. This may be explained by the fact that in addition to the solubilisation of AX, the exogenous (added) xylanase decreased the MW of the water-extractable AX in the WM bread. Presumably, the specificity and efficiency of the exogenous xylanase was so high that solubilised AX was degraded. Although the sourdough WM bread contained the highest amount of high MW water-extractable AX, the amount of total water-extractable AX in the sourdough WM bread was similar to that in the WM bread and in the white wheat bread. Thus, it is unlikely that water-extractable AX in the sourdough WM bread contributed to the observed low postprandial glucose and insulin responses in the present study.

Pre-test fasting and physical activity were controlled and strict inclusion criteria were used for the study subjects in the present study to decrease the inter- and intraindividual variation of the postprandial responses from visit to visit. However, we did not perform an oral glucose tolerance test when recruiting the subjects, which may have led to heterogeneity in the state of their postprandial glucose tolerance. Though we used a cross-over design, more detailed knowledge might have been arisen from glucose and insulin responses if the number of subjects had been larger.

According to the present results, wholemeal wheat bread made of peeled kernels and produced by sourdough fermentation lowers postprandial glucose and insulin responses compared with white wheat bread. Proteolysis taking place in sourdough fermentation may be a contributory factor, but more studies are needed to establish the actual underlying mechanism. The development of breads with low glycemic response and high wholemeal content is important so that the repeated high postprandial responses can be avoided and the nutrients in the grain preserved. Our present results indicate that the sourdough WM bread has an additional health promoting value in subjects with insulin resistance by lowering postprandial responses.
6 Effects of rye and whole wheat versus refined cereal foods on metabolic risk factors: a randomized controlled two-centre intervention study² (Study II)

6.1 SUMMARY

Background & aims: Intervention studies investigating the effects of wholegrain intake on glucose and insulin metabolism have provided conflicting results. Aim of this study was the evaluation of glucose and insulin metabolism in response to long-term consumption of rye and whole wheat compared with a diet containing the same amount of refined cereal foods, in individuals with metabolic syndrome form two European locations (Kuopio-Finland/Naples-Italy).

Methods: 149 individuals of both genders, age range 40-65 years with metabolic syndrome, were recruited to this study with parallel groups. After a 2-4 week run-in period, participants were assigned to a diet based on wholegrain (wholegrain group) or on refined cereal products (control group), each one for a duration of 12 weeks. Peripheral insulin sensitivity, assessed by FSIGT, lipids and inflammatory markers were measured before and at the end of intervention.

Results: 61 participants in the control group and 62 in the wholegrain group completed the dietary intervention. Compliance to the two diets was good. At the end of the intervention, insulin sensitivity indices and secretion (SI, QUICKI, DI, dAIRG) and lipids and inflammatory markers did not change significantly in the wholegrain and control groups as compared with baseline and no differences between the two groups were observed.

Conclusions: Wholegrain cereal foods consumption compared with refined cereals for 12 weeks did not affect peripheral insulin sensitivity.

6.2 INTRODUCTION

A large body of evidence from observational studies has shown that habitual consumption of cereal fibre and wholegrain foods is consistently associated with reduced risk of type 2 diabetes, metabolic syndrome and CVD (6). The protective effects of wholegrain cereals against type 2 diabetes and CVD have been attributed to a synergistic action of their components such as dietary fibre, vitamins and other molecules with antioxidant properties, phytoestrogens and micronutrients on several biological functions. However, the biological mechanisms responsible for the health effects of wholegrain are still unclear. Findings from epidemiological studies suggest that the benefits of wholegrain intake on human health are related to improved body weight, insulin sensitivity, lipid metabolism,

inflammation and antioxidant activity. Since insulin resistance is a key factor in the pathogenesis of type 2 diabetes and CVD, the reduced risk for type 2 diabetes and CVD observed in wholegrain consumers could be mediated by an improvement in insulin resistance. As a matter of fact, increased consumption of wholegrain was associated with higher insulin sensitivity, lower fasting insulin concentration, and lower 2-hour glucose concentration after an OGTT in many observational studies (29,31,32,34).

However, so far the results of intervention studies investigating the effects of wholegrain intake on glucose and insulin metabolism have provided conflicting results. Among the clinical trials with positive findings on insulin metabolism, a study in hyperinsulinemic overweight individuals showed that a 6-week period with a wholegrain rich diet, composed of 80% wheat, reduced fasting plasma insulin levels and improved insulin resistance as compared with a refined cereal diet (81); two other studies reported that high-fibre rye bread consumption compared with refined wheat bread significantly increased the first-phase of insulin secretion, suggesting an improved beta cell function (90), reduced fasting insulin levels and 24-h urinary C-peptide excretion (88). Other intervention studies observed benefits of wholegrain consumption on plasma cholesterol concentrations (84,94), systolic blood pressure levels (87), abdominal fat and hs-CRP (85,88) but no effect on glucose or insulin metabolism. Finally, some trials did not observe any effect of wholegrain consumption on either insulin sensitivity or metabolic abnormalities, or on inflammatory and oxidative status (83,89,95). These contradictory results could be related to either different wholegrain cereals used and/or differences in the pathophysiology of glucose metabolism in the participants in these studies, as well as to differences in approaches used to measure glucose and insulin metabolism (74).

The primary aim of our study was to evaluate differences in glucose and insulin metabolism, as assessed by FSIGT (frequently sampled intravenous glucose tolerance test) in response to longterm consumption of rye and wholegrain cereal products as compared with a diet containing the same amount of refined cereal foods, in individuals with the metabolic syndrome. The participants were recruited in two European locations (Kuopio, Finland and Naples, Italy) with different assortment of whole-grain products, food culture, genetic and life-style backgrounds. The secondary aim was to investigate the effects of this type of diet on lipid metabolism in inflammatory markers.

6.3 SUBJECTS AND METHODS

6.3.1 Population
One hundred and forty six individuals (85 from Kuopio and 61 from Naples) of both genders, age range 40-65 years with the metabolic syndrome, were recruited to participate in the dietary intervention. At screening, health status and medical history of the participants were examined by interview, clinical examination and routine laboratory tests (glucose, lipids, haemoglobin and liver, kidney and thyroid functions). In addition, a 75 g OGTT was carried out to evaluate glucose tolerance and exclude those with undiagnosed diabetes. The diagnosis of metabolic syndrome was based on the National Cholesterol Education Program Criteria (200). Individuals were excluded if they were diagnosed with
diabetes and/or renal failure (serum creatinine > 1.5 mg/dl), liver abnormalities (ALT/AST ratio two times above normal values), anaemia (Hb < 12 g/dl), any other chronic disease or if they used any drug able to influence glucose and lipid metabolism and inflammation (corticosteroid hormones other than inhaled corticosteroids, hypolipidemic or/and anti-inflammatory drugs); however, in the Kuopio study centre the use of cholesterol lowering medications (statins) was allowed.

All participants gave their written informed consent to the study which was approved by the Ethics Committee at the Kuopio University hospital and at the “Federico II” University of Naples.

6.3.2 Study design
The study was based on a randomized, controlled, parallel group design and consisted of a 2-4 week run-in period, during which the participants were stabilized on their own diet, and on 12-wk test period (Fig. 6.1). At the end of the run-in period, the participants were randomly assigned to one of two groups: one group consumed a diet based on wholegrain cereal products, most of them with a low postprandial glucose and/or insulin response (wholegrain group), and the other group consumed a diet based on refined cereal products (control group). The randomization was carried out separately at each centre with stratification for sex, age (5 years) and BMI (25-30, 30-35 kg/m²) by use of random allocation software. Allocation was carried out by personnel not involved in the study; therefore the investigators and the dieticians were aware of the group allocation of the participants only after the randomization process had been performed. During the study, participants were advised not to change their body weight and lifestyle habits such as exercise and alcohol consumption and not to change their medications unless necessary.

![Figure 6.1. Study design.](image)
At baseline and at 4, 8 and 12 weeks during the intervention, participants underwent clinical investigations including measurements of body weight, waist circumference and blood pressure; fasting blood samples were taken after a 12-hour overnight fast for biochemical measurements. After the run-in and at the end of the intervention, participants underwent FSIGT (208); in addition, BIA (bioelectrical impedance analysis) was performed to evaluate body composition.

6.3.3 Experimental diets
Participants were encouraged not to change their habitual meat, dairy products, eggs, fish, fruit, vegetable and fat intake during the study; the only difference between the wholegrain and the control diet was the inclusion of a fixed amount of wholegrain or refined cereal products as the main carbohydrate source. The wholegrain diet in Naples was based on wholegrain products including whole wheat bread (plus some endosperm rye bread), whole wheat pasta, barley kernels, wholegrain oat biscuits and breakfast cereals (all bran sticks and flakes) while the control diet contained commercial products based on refined cereals such as wheat bread, rice, pizza, cornmeal porridge, and breakfast cereals (rice krispies). The wholegrain bread consumed by the Neapolitan participant was 90 % sourdough whole wheat bread and 10 % endosperm rye bread. In Naples the average GI was 46 % for the wholegrain diet and 72 % for the control diet.

In Kuopio, the wholegrain and control diets were aimed to include 20-25 % of the total daily energy intake as study breads. In the wholegrain diet, the type of bread consumed by the Kuopio participants was 50 % commercial wholegrain rye bread, 40 % endosperm rye bread, and 10 % sourdough whole wheat bread. In addition, participants in Kuopio were advised to replace their habitual potato consumption with 210 g dry weight of whole wheat pasta per week, and were given whole oat biscuits for snacks. In the control group, the participants consumed commercial refined wheat breads, and only 1-2 small portions of rye products were allowed daily.

The wholegrain products used were defined as containing a minimum of 51 % wholegrain per dry substance, including the starchy endosperm, germ, and bran, mainly in milled form (11). Commercial wholegrain rye breads were made with 100 % wholegrain flour, and the endosperm rye bread with 100 % endosperm rye flour. Both the wholegrain rye bread and the endosperm rye bread were shown to produce a low postprandial insulin response (62). The sourdough whole wheat bread was shown to have beneficial postprandial glucose and insulin responses (209). Furthermore, both the Finnish and Italian sourdough whole wheat breads contained high amount of bioactive compounds present in bran like ferulic acid, betaine, choline and alkylresocinols (data not shown). In order to improve the adherence to the two experimental diets, the test products in both diets were provided free of charge to the participants in amounts sufficient to cover their household consumption for the whole duration of the study. For both diets cereals products represented about 60-80 % of the daily carbohydrate intake; the remaining 20-40 % of carbohydrates was provided by fruits and vegetables according to participants’ usual dietary habits. The intake of sugar and sugar-sweetened drinks was minimal (< 30 g/day)
The diets were controlled for energy intake to maintain body weight of participants stable during the whole period of the intervention.

6.3.4 Dietary assessment
Compliance with the experimental diets was assessed using a 4-d food records in Kuopio and 7-d food records in Naples during the run-in period, i.e. before starting the intervention, and at the 4th, 8th and 12th week of the intervention, to evaluate the energy intake and the nutrient composition of the diets followed by each participant (Fig. 6.1) (Table 6.1). Four-day food records included one weekend day. All 4-d food records were analysed with the MICRONUTRICA program version 2.0 (Finnish Social Insurance Institute, Turku, Finland), which utilizes a database of Finnish foods. All 7-d food records obtained in the Italian cohort were analysed by a computerized program using the food database of the Italian National Institute for Foods and Nutrition. If any of the foods recorded did not match a product already included in the database, the nutrient values declared by the producer were added to the database. For foods whose nutrients were not declared by the producer, the database value for the most similar product was used. In addition to food records, the intake of test products was followed by daily questionnaires filled in by the subjects in Kuopio and by counting the unused test products in Naples.

<table>
<thead>
<tr>
<th>Table 6.1. Energy intake and diet composition at baseline and at the end of the intervention</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control group (n=61)</strong></td>
</tr>
<tr>
<td><strong>Baseline</strong></td>
</tr>
<tr>
<td>Energy (kcal/d)</td>
</tr>
<tr>
<td>CHO (%)</td>
</tr>
<tr>
<td>Protein (%)</td>
</tr>
<tr>
<td>Total fat (%)</td>
</tr>
<tr>
<td>SAFA (%)</td>
</tr>
<tr>
<td>MUFA (%)</td>
</tr>
<tr>
<td>PUFA (%)</td>
</tr>
<tr>
<td>Cholesterol (mg/d)</td>
</tr>
<tr>
<td>Total fibre (g/d)</td>
</tr>
<tr>
<td>Cereal fibre (g/d)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mean ± SEM (all such values); <sup>b</sup> p<0.02 Paired sample t-test (12-week versus baseline); <sup>c</sup> Treatment effects (wholegrain versus control) were evaluated by GLM where study centre and baseline values were indicated as covariates, p<0.05

The energy and nutrient composition of wholegrain and refined wheat products employed in this trial were measured directly by the manufacturer. All participants received written and oral instructions given by the dietician or nutritionist concerning the diets to follow during the intervention and were supplied with recipes indicating how the products could be used in the best way to ensure good adherence to the diets. Weekly or biweekly the participants visited the clinic to collect new food products. During the visits for the clinical and body weight measurements at 4-week intervals, the participants returned the food records and questionnaires and were encouraged to continue on the study diet.

Plasma total AR concentration, a biomarker of wholegrain wheat and rye intake (69), was measured at baseline and at the end of the intervention in both groups in order to evaluate the compliance to the experimental diets.
6.3.5 Blood pressure, anthropometric and body composition measurements

Blood pressure was measured in the supine position in a standardized way after 5-10 min rest with an automatic sphygmomanometer. Body weight was measured on the same calibrated beam balance scale throughout the study. Waist circumference was measured halfway between the lowest rib and the iliac crest. Body composition was measured by bioelectrical impedance (BIA 101S with BODYGRAM software; Akern Srl Bio research, Florence, Italy).

6.3.6 Clinical test and laboratories analyses

Blood samples were drawn after a 12-hour overnight fast, from an antecubital vein for the measurements of plasma glucose, insulin, lipid, and inflammatory markers. Peripheral insulin sensitivity was assessed by FSIGT. A glucose dose of 300 mg/kg body weight was given intravenously followed by a bolus of 0.03 U/kg of insulin injected after 20 min. Blood samples were frequently collected for 3 h for the measurement of plasma glucose and serum insulin concentrations, utilized to calculate the insulin sensitivity index $\text{SI} (10^4 \text{ min}^{-1} /\text{µU/ml})$ (180,210). Insulin sensitivity at fasting was evaluated by the unitless QUICKI index. First-phase insulin response was evaluated as the average insulin concentration from 2 to 10 min and expressed as $\text{dAIRG (µU/ml)}$. The disposition index, DI, that measures the ability of the beta cell to increase its secretion to compensate for insulin resistance, was calculated as $\text{SI}^*\text{dAIRG}$ (78).

In Naples, plasma insulin concentrations were measured by an enzyme-linked immunosorbent assay (ELISA) for the specific determination of biologically active insulin (DAKO Insulin, DAKO Diagnostics, Ely, UK). Plasma glucose, cholesterol, and triglycerides were assayed by enzymatic colourimetric methods (ABX Diagnostics, Montpellier, France; Roche Molecular Biochemicals, Mannheim, Germany; Wako Chemicals GmbH, Neuss, Germany, respectively) on a Cobas Mira autoanalyzer (ABX Diagnostics, Montpellier, France). HDL cholesterol was isolated from plasma by a precipitation method with a sodium phosphotungstate and magnesium chloride solution and measured by the same enzymatic colourimetric method utilized for the analysis of total cholesterol. The LDL cholesterol concentration was calculated according to the formula of Friedwald.

In Kuopio, serum insulin was analysed with a chemiluminescent immunoassay (Advia Centaur, Siemens Medical Solution Diagnostics, Tarrytown, NY, USA), plasma glucose was analysed by the glucose hexokinase method (Konelab System Reagents; Thermo Fisher Scientific, Vantaa, Finland), and serum total, LDL-, and HDL-cholesterol, and triglycerides were analysed using commercial kits (Thermo Electron, Vantaa, Finland).

The inflammatory markers (hs-CRP, TNF-α, IL-6, IL-1ra) were determined in Germany at University of Ulm, in the laboratory of Department of Internal Medicine II-Cardiology, as described by de Mello et al (211). Measurements were done on plasma EDTA samples. Plasma samples were analysed for AR homologues C17:0-C25:0 according to a gas chromatographyemass spectrometry-single ion monitoring method, using molecular ions for quantification (212). Samples were divided randomly in 17 batches and analysed in singlets. In each batch, five replicates of a control sample were included randomly in the
sequence. The within- and between-day batch variation, determined as the coefficient of variation, was < 10%.

Laboratory analyses were performed blind in respect to the assigned treatment.

6.3.7 Statistical analyses
The main outcome variable in the statistical analysis was insulin sensitivity. The effects of dietary intervention on insulin sensitivity were analysed on 111 of 123 participants completing the study. On the basis of previous studies, a sample size of 120 individuals was calculated to detect a 20% difference in $S_i$ between the two groups with 0.05 significance level and 80% power (type II error = 0.1), assuming a 15% drop-out rate.

Results for continuous variables were presented as mean ± standard error of means (mean ± SEM), unless otherwise stated. Variables with skewed distributions by Shapiro-Wilks test were normalized with logarithmic or a square root transformation and were reported as median (interquartile range).

Energy intake and nutrient composition at baseline and during the intervention were calculated from the food records; the intakes during the intervention were expressed as mean of three food records filled in at 4, 8, and 12 weeks.

A general linear model (GLM) for repeated measures was used to evaluate differences between the groups (calculated as change of the parameter between 12-week and baseline and indicated as $\Delta$) during the intervention including the centre (Naples/Kuopio) and baseline variables as covariates. GLM for univariate analysis was used to assess the difference in the relative change (calculated as the change from baseline) of insulin sensitivity and insulin secretion indices between the groups with the centre and baseline variables as covariates. The effects of dietary intervention on insulin sensitivity between the groups were also analysed in a subgroup of participants having an $S_i$ index below the median value [< 2.8*10⁴ min⁻¹/(μU/ml)] at baseline. A paired-samples t-test was used to examine the changes compared with baseline in variables within each group. For all analyses, the level of statistical significance was set at $p = 0.05$ (two tails). Data were analysed with SPSS for Windows 11.5 (SPSS Inc., Chicago, IL).

6.4 RESULTS

6.4.1 Baseline characteristics of the participants
One hundred and twenty three participants (69 in Kuopio and 54 in Naples) completed the dietary intervention: 61 individuals (29M/32F) in the control diet group and 62 individuals (29M/33F) in the wholegrain diet group, while 14 individuals (18.7%) allocated in the control group and 9 (12.6%) in the wholegrain group dropped out because of limited time resources due to work or family-related problems. Of the participants completing the study, 55% had IFG and 27% had IGT at baseline. Furthermore, 92% presented high waist circumference, 52% low HDL cholesterol levels, 34% high fasting plasma triglyceride levels, 65% high systolic and 57% high diastolic blood pressure levels.
Clinical characteristics of participants are reported in Table 6.2. At the baseline, the wholegrain and control groups were similar with respect of age, body weight, BMI, waist circumference, body composition, blood pressure levels and fasting plasma concentrations of glucose, insulin, lipid, and ARs. The two groups were not different at baseline for insulin sensitivity (S1 and QUICKI) and for beta cell function (dAIRG) (Table 6.3). There were no differences in fasting plasma concentrations of the inflammation markers hs-CRP, IL-6, IL-1ra, and TNF-α at baseline (Table 6.4).

6.4.2 Dietary compliance
Compliance for the wholegrain and the control diets was good. During the intervention, both groups reported to consume the portions of the breads and wholegrain and/or refined cereal based products as advised. In the wholegrain group, the mean daily intake of the test breads in Kuopio was 185 g (of which commercial wholegrain rye bread (mean ± SD) 87 ± 26 g, endosperm rye bread 73 ± 21 g, and sourdough wholewheat bread 26 ± 4 g) and in Naples was 153 ± 44 g (of which sourdough wholewheat bread 139 ± 35 g, endosperm rye bread 14 ± 4 g). In the control group, the mean daily intake of refined wheat breads was 197 ± 46 g and 136 ± 47 g in Kuopio and Naples, respectively. In addition, in Naples the daily consumption of whole wheat pasta was on average 85.6 ± 22.8 g in the wholegrain group and that of refined wheat pasta plus rice plus pizza was on average 78 ± 19.0 g in the control group.

At baseline, the energy intake and nutrient composition of the diets were similar between the wholegrain and the control groups (Table 6.1). Compared with baseline, both the wholegrain and control groups increased their energy intake (mean value of dietary records at 4, 8 and 12 wk) during the intervention period (p = 0.001). However, the proportional nutrient composition did not change in the control group whereas slightly higher protein and PUFA intakes (p < 0.05) were observed in the wholegrain group (likely due to the higher protein and PUFA content in wholegrains than in refined grains). As expected, the wholegrain group significantly increased the intake of total and cereal fibre (p < 0.05); the differences as compared with the control group being 12.8 and 13.8 g/day, respectively; conversely, in the control group total and cereal fibre did not change after the intervention (Table 6.1).

Fasting plasma ARs concentrations increased significantly in the wholegrain diet group as compared with baseline and decreased in the control diet group with a significant difference between the two groups at the end of the intervention (+47 versus -28 nmol/l; p < 0.0001, GLM analysis) (Table 6.2). In particular, fasting plasma ARs concentrations increased in the wholegrain group as compared with baseline both in Kuopio (106.4 ± 89.3 versus 93.2 ± 69.8 nmol/l) and in Naples (140.2 ± 102.0 versus 52.0 ± 62.3 nmol/l) and decreased in the control group both in Kuopio (36.4 ± 26.8 versus 72.1 ± 52.1 nmol/l) and in Naples (43.7 ± 38.0 versus 63.4 ± 58.4 nmol/l).
<table>
<thead>
<tr>
<th></th>
<th>Control group (n=61)</th>
<th></th>
<th>Wholegrain group (n=62)</th>
<th></th>
<th>p for Δ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>12-Week</td>
<td>Δ</td>
<td>Baseline</td>
<td>12-Week</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>87.8 ± 15.8(^a)</td>
<td>87.8 ± 16.1</td>
<td>0.03(^b)</td>
<td>88.6 ± 15.8</td>
<td>87.9 ± 18.3</td>
</tr>
<tr>
<td>BMI (kg/m(^2))</td>
<td>31.3 ± 4.4</td>
<td>31.3 ± 4.5</td>
<td>0.00</td>
<td>31.6 ± 4.6</td>
<td>31.0 ± 5.8</td>
</tr>
<tr>
<td>WC (cm)</td>
<td>105.8 ± 10.7</td>
<td>105.8 ± 10.8</td>
<td>0.04</td>
<td>106.7 ± 13.0</td>
<td>106.4 ± 12.8</td>
</tr>
<tr>
<td>Lean mass (kg)</td>
<td>56.9 ± 12.3</td>
<td>56.6 ± 12.2</td>
<td>-0.3</td>
<td>56.3 ± 11.3</td>
<td>55.7 ± 11.3</td>
</tr>
<tr>
<td>Fat mass (kg)</td>
<td>30.9 ± 9.8</td>
<td>31.2 ± 10.3</td>
<td>0.3</td>
<td>32.3 ± 9.3</td>
<td>32.2 ± 9.4</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>135 ± 14</td>
<td>130 ± 17</td>
<td>-5</td>
<td>133 ± 15</td>
<td>128 ± 15</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>86 ± 8</td>
<td>82 ± 9</td>
<td>-4</td>
<td>84 ± 9</td>
<td>81 ± 9</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>6.06 ± 0.56</td>
<td>6.00 ± 0.61</td>
<td>-0.06</td>
<td>5.89 ± 0.56</td>
<td>5.94 ± 0.56</td>
</tr>
<tr>
<td>Insulin (pmol/l)</td>
<td>93.1 ± 45.1</td>
<td>93.1 ± 47.2</td>
<td>0.001</td>
<td>93.8 ± 52.8</td>
<td>103.5 ± 55.6</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>1.52 ± 0.72</td>
<td>1.56 ± 0.65</td>
<td>0.03</td>
<td>1.58 ± 1.20</td>
<td>1.65 ± 0.86</td>
</tr>
<tr>
<td>Cholesterol (mmol/l)</td>
<td>5.28 ± 0.93</td>
<td>5.31 ± 0.90</td>
<td>0.03</td>
<td>5.15 ± 1.09</td>
<td>5.25 ± 1.14</td>
</tr>
<tr>
<td>HDL-C (mmol/l)</td>
<td>1.14 ± 0.31</td>
<td>1.16 ± 0.31</td>
<td>0.02</td>
<td>1.16 ± 0.36</td>
<td>1.16 ± 0.34</td>
</tr>
<tr>
<td>LDL-C (mmol/l)</td>
<td>3.41 ± 0.80</td>
<td>3.41 ± 0.78</td>
<td>0.001</td>
<td>3.26 ± 0.98</td>
<td>3.31 ± 0.98</td>
</tr>
<tr>
<td>Total ARs (nmol/l)</td>
<td>68 ± 55</td>
<td>40 ± 32(^d)</td>
<td>-28</td>
<td>75 ± 69</td>
<td>122 ± 96(^d)</td>
</tr>
</tbody>
</table>

\(^a\) Mean ± SD (all such values)  
\(^b\) Δ = change of parameter between 12-week and baseline (all such values)  
\(^c\) Treatment effects (wholegrain versus control) were evaluated by GLM with the centre and baseline variables included as covariates  
\(^d\) p<0.02 Paired sample t-test (12-week versus baseline)
The nutrient intake of the participants in the Kuopio (n = 69) and Naples (n = 54) study centres was compared at baseline to evaluate any differences of the background diets in the two intervention sites. As compared with Kuopio, in Naples the intake of carbohydrates (50 ± 0.7 versus 44 ± 0.6 E %) and monounsaturated fatty acids (14.6 ± 0.4 versus 10.7 ± 0.3 E %) was higher (p < 0.05) while the intake of proteins (17.1 ± 0.4 versus 19.1 ± 0.4 E %), saturated fatty acids (9.8 ± 0.3 versus 12.3 ± 0.3 E %), polyunsaturated fatty acids (4.2 ± 0.2 versus 5.8 ± 0.2 E %), and cereal fibre (8.9 ± 0.5 versus 13.8 ± 0.7 g/day) was lower (p < 0.05). These differences in the background diet were maintained during the intervention (data not shown). Due to the different food cultures, the baseline diet in Kuopio was higher in cereal fibre than that in Naples because of the habitual intake of high fibre wholegrain rye bread in Kuopio. During the intervention, in Kuopio, about 40 % of the habitual intake of wholegrain rye bread was replaced in the wholegrain diet with endosperm rye bread containing less fibre (7 % as compared with 10-14%, respectively). On the contrary, in Naples, during the intervention the participants in the wholegrain diet group replaced the habitual refined grain consumption with wholegrain products.

6.4.3 Effects of dietary intervention on anthropometric and metabolic parameters
The mean body weight, BMI, waist circumference, fat mass and lean fat mass and systolic and diastolic blood pressure levels did not change during the intervention period in either group (Table 6.2). Before and at the end of the intervention, BMI was 31.6 ± 4.6 versus 31.0 ± 5.8 kg/m² in the wholegrain diet group and 31.3 ± 4.4 versus 31.3 ± 4.5 kg/m² in the control diet group; the waist circumference was 106.7 ± 13.0 versus 106.4 ± 12.8 cm in the wholegrain and 105.8 ± 10.7 versus 105.8 ± 10.8 cm in the control diet.

No effects of the wholegrain and control diet on fasting plasma concentrations of glucose, insulin and lipids were observed at the end of the intervention period (Table 6.2), as well as at 4 and 8 weeks (data not shown); however, fasting plasma insulin concentrations tended to be higher in the wholegrain group.

6.4.4 Effects of dietary intervention on insulin sensitivity and insulin secretion
At the end of the intervention, S_i, QUICKI, dAIRG and DI did not change significantly in the test and control groups as compared with baseline; no significant differences between the two groups were observed at the end of the intervention period (Table 6.3) (data were available in 54 and 57 subjects in the control and wholegrain groups, respectively). An additional analysis was performed on a subgroup of 54 participants (24 in the control group and 30 in the wholegrain group) who were more insulin resistant with S_i below the median value [< 2.8* 10^4 min/(µU/ml)] at baseline. Compared with baseline, in this subgroup S_i and QUICKI did not change after the wholegrain diet (S_i: 1.8 ± 0.1 versus 2.2 ± 0.2; QUICKI: 0.34 ± 0.004 versus 0.34 ± 0.004) and the control diet (S_i: 1.7 ± 0.1 versus 1.9 ± 0.2; QUICKI: 0.34 ± 0.004 versus 0.34 ± 0.004); no differences between the two groups were observed with respect to the changes from baseline between the two intervention groups.

6.4.5 Effects of dietary intervention on plasma inflammatory markers
Plasma concentrations of hs-CRP, IL-6, IL-1ra, and TNF-α did not change during the intervention, and did not differ between the test and the control group at the end of the intervention (Table 6.4).
Table 6.3. Markers of insulin sensitivity and insulin secretion at baseline and at the end of the intervention

<table>
<thead>
<tr>
<th></th>
<th><strong>Control group</strong> (n=54)^a</th>
<th></th>
<th><strong>Wholegrain group</strong> (n=57)</th>
<th>p for Δ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>12-Week Δ %</td>
<td>Baseline</td>
<td>12-Week Δ %</td>
</tr>
<tr>
<td>S_1</td>
<td>3.32 ± 0.26^b</td>
<td>3.18 ± 0.22</td>
<td>-4.2c (-18; +5)^d</td>
<td>2.97 ± 0.20</td>
</tr>
<tr>
<td>QUICKI</td>
<td>0.37 ± 0.006</td>
<td>0.36 ± 0.006</td>
<td>-1.39 (-2.6; -0.2)</td>
<td>0.36 ± 0.007</td>
</tr>
<tr>
<td>DI</td>
<td>146 ± 17</td>
<td>160 ± 21</td>
<td>+9.6 (-14; +24)</td>
<td>140 ± 17</td>
</tr>
<tr>
<td>dAIRg (2-10 min)</td>
<td>54.2 ± 5.2</td>
<td>55.7 ± 5.4</td>
<td>+2.8 (-8.2; +10.4)</td>
<td>61.4 ± 8.4</td>
</tr>
</tbody>
</table>

^a For 7 participants in the control group and 5 participants in the wholegrain group it was not possible to perform the FSIGT either at baseline or at follow-up for technical reasons

^b Mean ± SEM (all such values)

^c Δ = change of parameters between 12-week and baseline (all such values)

^d 95% C.I for Δ percentage (all such values)

^e Treatment effects (wholegrain versus control) were evaluated by GLM with the centre and baseline variables included as covariates

Table 6.4. Concentrations of plasma inflammatory markers at baseline and at the end of the intervention

<table>
<thead>
<tr>
<th></th>
<th><strong>Control group</strong> (n=61)</th>
<th></th>
<th><strong>Wholegrain group</strong> (n=62)</th>
<th>p for group effect</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median (interquartile range)</td>
<td>Median (interquartile range)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Baseline</td>
<td>12-Week</td>
<td>Baseline</td>
<td>12-Week</td>
</tr>
<tr>
<td>hs-CRP^a (mg/dl)</td>
<td>1.95 (0.96; 2.56)</td>
<td>1.74 (1.04; 2.95)</td>
<td>1.95 (0.74; 4.12)</td>
<td>1.36 (0.62; 3.34)</td>
</tr>
<tr>
<td>IL-6 (pg/ml)</td>
<td>1.41 (0.84; 2.21)</td>
<td>1.43 (1.07; 2.11)</td>
<td>1.42 (1.01; 2.32)</td>
<td>1.54 (1.12; 2.23)</td>
</tr>
<tr>
<td>IL-1ra (pg/ml)</td>
<td>251 (193; 330)</td>
<td>239 (190; 379)</td>
<td>300 (214; 518)</td>
<td>298 (175; 386)</td>
</tr>
<tr>
<td>TNF-α (pg/ml)</td>
<td>0.62 (0.43; 1.05)</td>
<td>0.63 (0.41; 0.90)</td>
<td>0.73 (0.50; 0.96)</td>
<td>0.68 (0.50; 0.94)</td>
</tr>
</tbody>
</table>

^a hs-CRP data on 49 participants in the control group and 52 participants in the wholegrain group, untreated with cholesterol lowering medications

^b Treatment effects (wholegrain versus control) on the change as Δ (12-week – baseline) were evaluated by GLM with the centre and baseline variables included as covariates
Since there were some individuals at the Kuopio study centre using cholesterol lowering medication (n=10 in the test and 10 in the control group), we also performed the statistical analysis after excluding these individuals. In addition, individuals with high baseline value of hs-CRP (>10mg/l) in Kuopio (n=1) and in Naples (n=1), both in the control group, were excluded as outliers. There was a trend for a decreased hs-CRP concentration at the end of the wholegrain diet [1.36 mg/dl (0.62; 3.34)] [median (interquartile range)] as compared with baseline [1.95 mg/dl (0.74; 4.12)] (p=0.08).

6.5 DISCUSSION

Twelve weeks consumption of rye and wholegrain wheat based diets compared with corresponding refined diets did not improve glucose and insulin metabolism nor lipid and inflammatory markers in this randomised, controlled, two-centre intervention study with Finnish and Italian individuals at risk of type 2 diabetes. These results are in line with those of previous interventions showing no effects of wholegrain consumption on the above mentioned markers (83,89) or on insulin sensitivity (84,85,87). However, the results of the present study are at variance with the findings of improved insulin sensitivity or first-phase insulin response observed in association with an increased consumption of whole wheat (81) or wholegrain rye products in some studies (88,90,92). The conflicting results on glucose and insulin metabolism in wholegrain intervention studies may be due to differences in the methodologies used for analysing glucose and insulin metabolism or to differences in to the amount and type of wholegrain products of experimental diets (Appendix table).

In this context, it is worth to underline that our study, using both an adequate sample size and a validated methodology for insulin sensitivity measurement, was unable to show any effect of wholegrain on glucose and insulin metabolism. In fact, peripheral insulin sensitivity was evaluated by FSIGT on a sample of 111 individuals, suitable to detect a clinically relevant effect (20 %) of wholegrain on insulin sensitivity with a low risk (0.1) for type II error. Any smaller effect may lack clinical relevance. However, since the test here employed to evaluate insulin sensitivity takes into account predominantly insulin effects at the level of muscle and adipose tissue, it cannot be completely ruled out the possibility of beneficial effects of wholegrain consumption on glucose and insulin metabolism at the splanchnic site and in the postprandial period.

No effects on the first-phase insulin secretion by either diet were observed in this study using the data from FSIGT, contrary to the study by Laaksonen et al (92) in which insulin secretion calculated from OGTT improved after the rye-pasta diet as compared with the oat-wheat-potato diet. However, the role of gastrointestinal hormones during an OGTT should also be taken into account when assessing the insulin response.

It is also to be considered that in our intervention the daily intake of cereal fibre from the wholegrain diet was lower (24.3 ± 0.9 g) than that consumed in the studies of Juntunen (29.7 ± 6.3 g (90) and Landberg (58 ± 7.0 g) (88) which, respectively, observed beneficial effects of wholegrain rye products on the acute insulin response and on the fasting insulin and C-
peptide concentrations. However, the daily cereal fibre intake in our intervention is in line with the amount associated to a lower risk of type 2 diabetes in epidemiological studies (6,213). In addition, our daily cereal fibre intake, compared with that very high reported in the Landberg study, is better tolerated by consumers and more sustainable in the long term.

Moreover, in our intervention, the wholegrain diet contained a variety of whole wheat and rye products accompanied by smaller amounts of oat and barley. Statistical analysis performed separately for the two centres, Kuopio and Naples, showed no difference in SI and insulin secretion between the two intervention groups in the participants of either centre, although in Kuopio the wholegrain group consumed 90% of daily bread as wholegrain and endosperm rye bread and in Naples as whole wheat bread. It is also noteworthy that the wholegrain diet had a lower GI than the control diet in Naples but not in Kuopio. Therefore, we could rule out that in our experiment the absence of any beneficial effect of wholegrain on measured glucose and insulin metabolism could depend on the type of grain (rye or wheat) and environment or culture.

In relation to the relationship between wholegrain consumption and markers of subclinical inflammation, it is worth stressing a reduction of plasma hs-CRP has been observed only when wholegrain and bran rye products were consumed and that the intake of endosperm rye bread is negatively correlated with concentrations of hs-CRP in plasma, as observed respectively by Landberg et al (88) and de Mello et al (211).

An additional strength in our intervention was that plasma AR was used as a biomarker for the intake of whole wheat and rye (69). The food records showed a significant increase in the cereal fibre intake in the wholegrain group and this was confirmed by an increase in plasma ARs concentrations, while conversely, ARs plasma levels were decreased in the control group. These data indicate a good adherence to the prescribed dietary treatments. The evaluation of the AR homologues in plasma confirmed a higher consumption of rye in Kuopio as compared with a higher consumption of wheat in Naples (data not shown).

Furthermore, during the intervention, participants, according to the study design, maintained stable their body weight, body fat composition and waist circumference that may represent confounding factors on insulin sensitivity and secretion (32,85,214). Our study has many strengths but also some limitations. In fact, it does not allow ruling out the possibility of a more relevant effect of wholegrain on glucose and insulin metabolism in individuals with more pronounced metabolic derangements. In addition, a more focused evaluation of insulin sensitivity at the liver site would probably be more appropriate to investigate the metabolic impact of wholegrain since the effects of wholegrain are probably mediated by mechanisms acting in the splanchnic region, predominantly in the postprandial period. Further limitation of the study is the treatment duration which could be too short to induce relevant modifications in metabolic parameters which interfere with plasma glucose and insulin regulation.

In conclusion, our randomized, well controlled two-centre intervention clearly shows that rye or wheat based wholegrain diets did not affect peripheral insulin sensitivity and other parameters of glucose metabolism in individuals with the metabolic syndrome. However, it
remains to be elucidated with intervention studies of appropriate duration and sample size whether wholegrain consumption is able to reduce the risk of type 2 diabetes or cardiovascular diseases as suggested by observational studies.
7 Intake of wholegrain and fiber-rich rye bread versus refined wheat bread does not differentiate intestinal microbiota composition in Finnish adults with metabolic syndrome\textsuperscript{3} (Study III)

7.1 ABSTRACT

Whole-grain (WG) foods rich in indigestible carbohydrates are thought to modulate the composition of the intestinal microbiota. We investigated in a randomized, parallel, 2-arm 12-wk intervention whether consumption of WG and fiber-rich rye breads compared with refined wheat breads affected the microbiota composition in Finnish individuals aged 60 ± 6 y with metabolic syndrome. Fecal samples from 51 participants (25 males, 26 females) before and after the intervention were processed for the microbiota analysis using a phylogenetic microarray and quantitative polymerase chain reactions targeting the 16S rRNA gene. The intake of whole grains calculated from food records was higher in the group consuming rye breads (75 g) than in that consuming refined wheat breads (4 g; P < 0.001), confirmed by fasting plasma alkylresorcinol concentrations, a biomarker of whole grain intake. The intestinal microbiota composition did not significantly differ between the groups after the intervention. However, we detected a 37 % decrease of Bacteroidetes (P < 0.05) in parallel to a 53 % decrease in the alkylresorcinol concentration (P < 0.001) in the group consuming refined wheat breads. In this group, the abundance of bacteria related to Bacteroides vulgatus, B. plebeius, and Prevotella tannerae decreased, whereas that of bacteria related to Collinsella and members of the Clostridium clusters IV and XI increased. In a multivariate regression analysis, the abundance of Bacteroides spp. was best explained by different fat compounds among dietary variables, whereas the main sugar-converting butyrate-producers were mostly associated with the intake of whole- and refined-grain bread and fiber. Our results indicate that the quality of grains has a minor effect on the intestinal microbiota composition in participants with metabolic syndrome and suggest that the dietary influence on the microbiota involves other dietary components such as fat.

7.2 INTRODUCTION

The amount and nature of ingested carbohydrates are assumed to affect the composition and activity of the intestinal microbiota that dominate the large intestine. A low-carbohydrate diet has been observed to significantly decrease the main butyrate producers, Roseburia spp. and Eubacterium rectale, compared with an isoenergetic high-protein or normal diet (139). However, the amount of indigestible carbohydrates that reaches the large

intestine is more likely to affect the microflora than the total carbohydrate content of diet per se. Whole grains are rich in various indigestible carbohydrates, including cellulose, arabinofuranose, β-glucan, and fructan. Arabinofuranose is one of the main dietary fibers in wheat and rye (56,63). Refined grains lack these compounds mainly due to the removal of the bran layer of the grain. Some intestinal bacteria can ferment arabinofuranose in model systems, but in vivo data in humans are scarce (215). Hence, the amount of whole-grain (WG) foods in the diet is expected to largely control the amount of fermentable substrates available for the large intestinal microflora. Furthermore, other nutrients and staple foods might also play a role in modifying the microflora composition, as suggested by cross-sectional studies that indicated an adaptation of specific bacterial groups to the content of complex carbohydrates, fat, and protein in the diet (216-218).

An increasing number of studies indicate that diet affects the development of metabolic disorders, such as type 2 diabetes, possibly via an effect on the intestinal microflora (7,17,219). However, there are only a limited number of human intervention studies relating WG food consumption with the intestinal microflora composition. A 2-wk, randomized, cross-over study reported slightly increased numbers of Clostridium leptum but showed no difference in Bifidobacteria or Bacteroides in 17 healthy participants when a diet based on whole grains was compared with one based on refined grains (96). Similarly, in another strictly controlled intervention study, a diet supplemented with wheat bran did not notably change the intestinal microflora composition (143). In contrast, Costabile et al. (142) observed that the numbers of Bifidobacteria increased after consumption of a WG wheat breakfast cereal compared with one based on wheat bran for 3 wk. While this result suggests that whole grains are more bifidogenic than wheat bran alone, no other differences were reported for the analyzed bacterial groups between the treatments. Recently, a randomized cross-over study showed that adding WG barley flakes to the diet for 4 wk increased the abundance of Bifidobacterium, Blautia, and Roseburia spp (146). Finally, a study entailing 69 participants with metabolic syndrome showed that a diet supplemented with a purified cereal fiber extract had no effect on the intestinal microflora composition or fermentation profile (147). Most of the inconclusive present results are based on targeted intestinal microflora analysis with a limited set of dominant or otherwise relevant bacterial groups. Thus, they do not provide a global view of the microflora during dietary change.

In summary, it has not yet been established whether or how the type of cereal foods affects intestinal microflora. Hence, we investigated the effects of refined low-fiber wheat bread and WG and high-fiber rye bread intake on the intestinal microflora composition in 51 participants with metabolic syndrome. The dietary difference was mainly achieved by changing the bread type in diet, i.e., by affecting the quality of one of the staple foods. We performed a comprehensive, deep, and high throughput analysis of the intestinal microflora composition using a phylogenetic microarray and additionally addressed associations of the intestinal microflora with nutrient and food intake.
7.3 METHODS

7.3.1 Participants and study design
Participants were recruited into a 12-wk, parallel, controlled dietary intervention study from the Kuopio area of Finland as previously described (86,211). Fifty-two participants in 2 intervention groups provided fecal samples. Of these, one participant was excluded because of diagnosed inflammatory bowel disease. Thus, 51 participants (25 males, 26 females) were studied for effects of diet on the intestinal microbiota composition and clinical variables and for associations between diet and microbiota.

Inclusion criteria for the participants were age 40–65 y, a BMI of 26–39 kg/m², and at least 3 other features of metabolic syndrome: impaired glucose tolerance (2-hour glucose 7.8–11.0 mmol/L) or impaired fasting glucose (glucose 5.6–6.9 mmol/L), waist circumference >102 cm (men) or >88 cm (women), fasting serum TG concentration >1.7 mmol/L, fasting serum HDL cholesterol concentration <1.0 mmol/L (men) or <1.3 mmol/L (women), and blood pressure >130/85 mm Hg or medication for hypertension. Exclusion criteria were: BMI >40 kg/m²; fasting serum TG concentration >3.5 mmol/L; fasting serum total cholesterol concentration >8 mmol/L; type 1 or 2 diabetes; abnormal liver, thyroid, or renal function; alcohol abuse [> 16 portions/wk (women) or > 24 portions/wk (men)], and inflammatory bowel disease. The participants were randomized to a rye bread (RB) diet (n = 27) or a refined white wheat bread (WWB) diet (n = 24). The dietary groups were matched for gender, BMI, age, and fasting plasma glucose concentration. The protocol for the study was approved by the Ethics Committee of the Hospital District of Northern Savo. Written informed consent was obtained from all participants.

7.3.2 Intervention diets
Participants in the RB group consumed rye breads with high-fiber content (7–15 %) and those in the WWB group consumed refined wheat breads with a low fiber content (4 %). Most of the total grain intake consisted of bread (aiming to cover 20–25 % of total energy intake). The test breads were chosen on the basis of our previous postprandial studies with rye and wholemeal wheat breads showing a beneficial low-insulin response (62,209). The breads in the RB group were a selection of commercial WG rye breads (50 % share of all the breads), endosperm rye bread (40 % share), and a wholemeal wheat bread (10 % share). In addition, the participants in the RB group were asked to consume wholemeal pasta [3.5 dL/wk (measured as uncooked)] and were given high-fiber oat biscuits for voluntary intake. In the WWB group, the test breads were a selection of commercial refined wheat breads and the intake of rye products was restricted to 1–2 portions/d. The participants were provided with the test products and advised by a registered dietician on the practical management of the diet. Assessment of dietary compliance was based on questionnaires where the participants recorded their consumption of the test products daily. Apart from the grain products, the participants’ habitual diet and lifestyle habits were not controlled but were advised to keep unchanged during the trial.

7.3.3 Dietary analyses
Participants filled in 4-d food records at baseline and at the end of the intervention (i.e., wk 11). Dietary data for the intake of nutrients and food groups was analyzed by MicroNutra software version 2.0 (Finnish Social Insurance Institute). Nutrient intake-based
dietary evaluation was complemented by calculating intake of food items. The food items were grouped into several categories such as WG breads, refined white breads, other grain products, vegetables, fruits, spreads, dairy products, meat, fish, and drinks (a more detailed description of the food groups is in Supplemental Table 1). In addition, intakes of grain fiber and WG ingredients (i.e., whole grains) were calculated from food records using data from food labels and common recipes.

7.3.4 Clinical measurements and biochemical analyses
Clinical variables were measured at baseline and at the end of the intervention. Measurements and analyses of fasting glucose, insulin, serum cholesterol fractions and TGs, and markers of glucose metabolism and inflammation were described earlier (86,211).

The plasma total alkylresorcinol (AR) concentration, a biomarker of WG intake (69,220), was analyzed at baseline and at the end of the intervention to evaluate the compliance to the intervention diets. Fasting plasma samples were analyzed for AR homologs C17:0–C25:0 according to a GC-MS–single ion monitoring method, using molecular ions for quantification (212). Total concentration of AR was calculated from the sum of homologs.

7.3.5 Compositional analysis of the intestinal microbiota
Fecal spot samples were collected by the participants at home at baseline and the end of the intervention. The samples were stored in −70°C after delivering to the Department of Clinical Nutrition either as cooled or being frozen in a home freezer (−18°C) for less than a day. Extraction of bacterial DNA from the fecal samples was performed using mechanical lysis (221). Compositional analysis of the intestinal microbiota was performed using the Human Intestinal Tract Chip (HITChip), a phylogenetic microarray produced by Agilent Technologies as previously described (181). In brief, the HITChip consists of >4800 oligonucleotide probes targeting 1033 distinct phylotypes based on the V1 and V6 hypervariable regions of the 16S rRNA (181). Phylogenic assignment of the probes and quality control of the HITChip array data have been described in detail elsewhere (181,221). The array data were first min-max normalized and then a background subtraction was carried out as in Zilliox et al. (222), using the mean background level plus 1 SD as noise threshold. All analyses were done on duplicate arrays and the data only passed the quality control when the Pearson’s coefficient was > 98 % (181). The amount of total bacteria and methanogenic archaea in the samples was determined with qPCR as previously described (223).

7.3.6 Statistical analyses
Statistical analyses were performed with scripts in R, version 2.15.1 (R Development CT. Vienna, Austria: R Foundation for Statistical Computing; 2012. R: A Language and Environment for Statistical Computing). All statistical analyses were carried out with a 10-base logarithm-transformed data.

For the HITChip, dietary, and clinical data, a comparison between the groups was carried out with the nlme package in R (224) using a linear mixed model, with subject modeled as a random effect, and time, intervention group, and their interaction as fixed effects. The contrasts were then estimated with the multcomp package (225). Pairwise t tests were used to estimate the significance of changes within group as well as to analyze the qPCR data.
After their estimation, all P values of fixed effects and different comparisons were subjected to Benjamini-Hochberg false discovery rate correction. The adjusted P values < 0.05 were regarded as significant. Statistical over-representation (enrichment) of genus-like groups among the significant phylotypes was tested using Fisher’s exact test (226). The dietary, clinical, and qPCR data are expressed as means ± SDs for variables with normal distribution and as median (minimum-maximum) for initially skewed distributions.

Hierarchical clustering of the HITChip microbiota profiles was carried out by using nonbackground-subtracted, oligo-level data using correlation as the distance measure and complete linkage clustering algorithm. Multivariate analysis was carried out with bootstrap aggregated (bagged) redundancy analysis (RDA) and partial least squares (PLSs) that allow identifying sets of covariates whose joint effect explains the dependent variable(s). RDA was applied to find sets of bacteria that separate dietary groups. PLS was used to find a set of nutrients or food groups explaining the variation of these sets of bacteria. In this setting, the WG variable was dropped out from analysis, because it was a control variable between the dietary groups. Overall associations between the microbiota and dietary variables were analyzed with PLS, where the dependent variable was the RDA component separating the 2 dietary groups in food intake data. Bootstrap aggregated (bagged) RDA and PLS were implemented as R scripts with 10,000 bootstrap data sets, using vegan package (227) to estimate the RDA or PLS solutions for each set and using Procrustes rotations to solve the rotational ambiguity. In PLS, R² (proportion of variation) was estimated with a bootstrap 0.632+ method (228). The latent dimensionality of PLS was set to 2. Missing values in the data matrices were imputed with a probabilistic principal component analysis (229). Biserial mid-correlations were used to correlate bacterial genus-like groups with nutrients and food groups. Here, a looser threshold for significance was employed for exploratory purposes by including correlations that had an > 80% chance of being true positives.

7.4 RESULTS

7.4.1 Characteristics of participants and diet
The participants were 60 ± 6 y of age at baseline and fulfilled the set criteria for metabolic syndrome as having a BMI of 31 ± 4 kg/m², fasting plasma glucose concentration of 6.1 ± 0.5 mmol/L, waist circumference of 111 ± 9 cm (men) or 101 ± 8 cm (women), and systolic or diastolic blood pressure of 140 ± 13 or 88 ± 8 mm Hg, respectively (53% of the participants had medication for hypertension). The markers of metabolic syndrome did not differ between the study groups, which were in line with the results from the original larger study group (86,211). The weight of the participants remained the same throughout the intervention (89 ± 14 kg). The intervention did not induce differences between the groups in the markers of glucose metabolism and inflammation, as also previously reported (86,211), although there was improvement in the high sensitivity C-reactive protein concentration within the RB group (211).

As expected based on the diet modification, the daily intakes of total and grain fiber and whole grains differed between the groups during the intervention (P < 0.05), such that the intakes were lower at the end of the intervention in the WWB group than in the RB group
(P < 0.001) (Table 7.1). Within the WWB group, the intake of total fiber, grain fiber, and whole grains decreased by 2, 3, and 51 g, respectively (P < 0.05). Within the RB group, the intake of grain fiber increased (P < 0.001) by 5 g and that of whole grains increased (P < 0.01) by 8 g, but there was no change in the total fiber intake. The fasting plasma concentration of AR differed between the groups during the intervention (P < 0.05) by decreasing 53% in the WWB group (P < 0.001) and being lower than in the RB group at the end of the intervention (P < 0.001), confirming the compliance of the participants regarding consumption of grain products.

During the intervention, the daily intake of energy similarly increased by 1040 kJ (P < 0.05) and 1390 kJ (P < 0.01) in the RB and WWB groups, respectively. However, the percentage of energy from the energy-yielding nutrients did not change, except for PUFA, 18:2n6, and 18:3n3, which decreased in the RB group (P < 0.05).

Table 7.1. Intake of nutrients and plasma alkylresorcinol concentrations at wk 0 and 11 in RB and WWB groups

<table>
<thead>
<tr>
<th></th>
<th>RB group (n=27)</th>
<th>WWB group (n=24)</th>
<th>P-interaction²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>wk 0</td>
<td>wk 11</td>
<td>wk 0</td>
</tr>
<tr>
<td>Energy intake, kJ/d</td>
<td>6830 ± 2220</td>
<td>7870 ± 2880*</td>
<td>7200 ± 1840</td>
</tr>
<tr>
<td>Carbohydrate, E %</td>
<td>46 ± 7</td>
<td>46 ± 9</td>
<td>48 ± 5</td>
</tr>
<tr>
<td>Protein, E %</td>
<td>19 ± 3</td>
<td>19 ± 3</td>
<td>19 ± 4</td>
</tr>
<tr>
<td>Fat, E %</td>
<td>33 ± 5</td>
<td>33 ± 7</td>
<td>31 ± 6</td>
</tr>
<tr>
<td>SAFA, E %</td>
<td>12.1 ± 2.2</td>
<td>12.1 ± 3.0</td>
<td>12.0 ± 2.8</td>
</tr>
<tr>
<td>MUFA, E %</td>
<td>11.3 ± 2.1</td>
<td>10.5 ± 3.6</td>
<td>9.9 ± 2.3</td>
</tr>
<tr>
<td>PUFA, E %</td>
<td>5.8 (3.6-10.1)</td>
<td>4.6 (1.3-9.6)*</td>
<td>5.2 (3.3-9.9)</td>
</tr>
<tr>
<td>18:2n6, E %</td>
<td>3.9 (2.5-7.3)</td>
<td>3.2 (1.0-8.5)*</td>
<td>3.6 (1.9-6.5)</td>
</tr>
<tr>
<td>18:3n3, E %</td>
<td>1.0 (0.4-1.4)</td>
<td>0.8 (0.2-1.3)*</td>
<td>0.7 (0.3-1.9)</td>
</tr>
<tr>
<td>Total fibre, g/d</td>
<td>24 (12-38)</td>
<td>24 (17-42)</td>
<td>21 (14-39)</td>
</tr>
<tr>
<td>Grain fibre, g/d</td>
<td>14 (3-32)</td>
<td>19 (15-38)**</td>
<td>13 (7-20)</td>
</tr>
<tr>
<td>Whole grains, g/d</td>
<td>67 (4-211)</td>
<td>75 (41-164)**</td>
<td>55 (17-101)</td>
</tr>
<tr>
<td>P-AR, nmol/l</td>
<td>94 (22-263)</td>
<td>83 (32-476)</td>
<td>51 (23-226)</td>
</tr>
</tbody>
</table>

1 Values are mean ± SDs or median (minimum-maximum). Different from RB at wk 11: *p<0.001 (linear mixed model with false discovery rate correction). Different from wk 0: *p<0.05, **p<0.01, ***p<0.001 (pairwise t-test with false discovery rate correction). AR, alkylresorcinol; E %, percentage of total energy intake; RB, rye bread; WWB, white wheat bread.

2 P-value for group x time interaction (linear mixed model with false discovery rate correction)

An analysis of the food groups showed differences mainly in the intake of grain products, as expected. The intake of WG breads decreased and that of refined white breads increased in the WWB group compared with the RB group (P < 0.001) (Table 7.2). In the RB group, the intake of endosperm rye bread, whole-meal pasta, and oat biscuits increased (P < 0.001). There were no other differences in the mean intake of food items between the groups during the intervention, but individual-specific differences were noted, reflecting the individuality in the habitual dietary patterns (Supplemental Table 2).
Table 7.2. Intake of grain foods at wk 0 and 11 in RB and WWB groups

<table>
<thead>
<tr>
<th></th>
<th>RB group (n=27)</th>
<th>WWB group (n=24)</th>
<th>P-interaction²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>wk 0</td>
<td>wk 11</td>
<td>wk 0</td>
</tr>
<tr>
<td>g/d</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WG breads</td>
<td>94 (0-267)</td>
<td>92 (50-157)</td>
<td>78 (15-111)</td>
</tr>
<tr>
<td>High-fibre breads</td>
<td>0 (0-75)</td>
<td>0 (0-17)</td>
<td>0 (0-180)</td>
</tr>
<tr>
<td>Refined white breads</td>
<td>15 (0-215)</td>
<td>0 (0-12)</td>
<td>46 (0-236)</td>
</tr>
<tr>
<td>Endosperm rye bread</td>
<td>0 (0-0)</td>
<td>60 (0-175)</td>
<td>0 (0-0)</td>
</tr>
<tr>
<td>Wholemeal pasta</td>
<td>0 (0-24)</td>
<td>12 (0-56)</td>
<td>0 (0-0)</td>
</tr>
<tr>
<td>Other pasta</td>
<td>0 (0-68)</td>
<td>0 (0-50)</td>
<td>0 (0-50)</td>
</tr>
<tr>
<td>Oat biscuit</td>
<td>0 (0-0)</td>
<td>8 (0-68)</td>
<td>0 (0-0)</td>
</tr>
<tr>
<td>Other WG products</td>
<td>17 (0-56)</td>
<td>2 (0-34)</td>
<td>7 (0-79)</td>
</tr>
<tr>
<td>Other grain products</td>
<td>41 (4-121)</td>
<td>13 (0-118)</td>
<td>52 (14-143)</td>
</tr>
</tbody>
</table>

¹Values are median (minimum-maximum). Different from RB at wk 11: *p<0.05, **p<0.001 (linear mixed model with false discovery rate correction). Different from wk 0: †p<0.05, ‡p<0.001 (pairwise t-test with false discovery rate correction). RB, rye bread; WG, wholegrain; WWB, white wheat bread
²P-value for group x time interaction (linear mixed model with false discovery rate correction)

7.4.2 Intestinal microbiota composition

The composition of the intestinal microbiota was analyzed by hybridizing the 16S rRNA amplicons on the HITChip phylogenetic microarray, which targets more than 1000 intestinal phylotypes covering most of the so-far-known diversity (181). Hierarchical clustering of the microbiota profiles was performed to gain an overview of the similarity of the total intestinal microbiota within and between the participants. Despite the change in diet, the microbiota had high individual specificity and temporal stability (within-subject Pearson correlation = 0.92 ± 0.03, with no difference between the groups). The microbiota composition of none of the participants changed to the extent that it would have hampered the clustering according to participant (Supplemental Fig. 1). The probabilistic principal component analysis that addressed the maximal variation in the HITChip data also did not show segregation of the samples by the intervention group or pre- and postintervention samples (data not shown). Based on qPCR analysis of 16S rRNA amplicons, the amount of total bacteria and methanogenic archaea was 11.7 ± 0.2 and 8.2 ± 1.2 log₁₀ genome copies/g feces, respectively. These values did not differ between the groups or before and after the intervention.

Linear models were applied to identify the bacteria whose relative abundance significantly differed between the groups or whose abundance significantly changed within a group during the intervention. Comparative analyses were performed on different phylogenetic levels by summing up the probe signal intensities to phylotype (species-like), genus-like, or phylum levels. The microbiota composition did not differ between the groups either at baseline or after the intervention, except for the phylotype Bryantella formatexigens, which was 16% higher in the WWB group at the end of the intervention (P = 0.04).
When analyzing the within-group effects, the microbiota composition changed in the WWB group. The participants’ microbiota showed a decrease (P < 0.05) of Bacteroidetes spp. paralleled by an increase in the members of Clostridium cluster IV (Firmicutes) as well as Collinsella and Atopobium spp. that belong to the Actinobacteria (Fig. 7.1). The relative proportion of Bacteroidetes phylum decreased 37% during the intervention. On the genus-like level, 9 taxa were enriched based on 55 phylotypes that showed a change in the WWB group (Supplemental Table 3). Among the enriched genera, Bacteroides plebeius and relatives (et rel.), B. vulgatus et rel. and Prevotella tannerae et rel., decreased, whereas mainly uncultured taxa belonging to Clostridium clusters IV (Clostridium leptum et rel., Clostridium cellulosi et rel., and Anaerotruncus colihominis et rel.) and XI (Anaerovorax odorimutans et rel.) as well as to Actinobacteria (Atopobium and Collinsella) increased (Fig. 7.1). The largest mean decrease was detected in B. vulgatus et rel. (19% decrease) and the largest increase in Clostridium cluster IV (17% increase for Clostridium cellulosi et rel.). In the RB group, the intervention diet did not change the relative abundance of any bacterial taxa (this was observed at all phylogenetic levels) (data not shown).

A substantial individuality characterized the microbiota responses. Although the changes in the majority of the participants in the WWB group were unidirectional, in some individuals, the implicated taxa did not respond at all or even changed to the opposite direction (Supplemental Fig. 2). On the other hand, the abundance of B. vulgatus that mostly contributed to the decrease of Bacteroidetes in the WWB group also decreased in about one-half of the participants in the RB group (Supplemental Fig. 3). Among the genus-like bacterial groups, Bifidobacterium spp. varied the most during the intervention (fold change per individual ranged from 0.17 to 9.12), but the degree of variation was independent of the dietary group.

Figure 7.1. Magnitude and direction of change in the 9 significantly enriched, genus-like, phylogenetic bacterial groups in the WWB group during the intervention. A higher phylogenetic level of each group is indicated on the left with order for Firmicutes and phylum for others. WWB, white wheat bread.
7.4.3 Associations of the intestinal microbiota with nutrient and food intake

In the RDA analysis of the HITChip data, the bacteria contributing to the separation between the diet groups were dominated by the members of Bacteroidetes (Supplemental Fig. 4), in line with the changes we identified within the WWB group. The abundance of 12 Bacteroides-Prevotella taxa differed or tended to differ (P = 0.01–0.10) between the diet groups [referred to as the Bacteroides cluster below, consisting of B. vulgatus (P < 0.05), P. tannerae (P < 0.05), B. intestinalis (P < 0.05), B. plebeius (P < 0.05), P. oralis (P < 0.05), P. ruminicola (P = 0.06), B. ovatus (P = 0.08), B. uniformis (P < 0.05), B. stercoris (P < 0.05), B. fragilis (P = 0.10), B. splachnicus (P = 0.10), and P. melaninogenica (P = 0.06)]. To find an explanation for the variation in the Bacteroides cluster, their joint abundance in relation to dietary variables was analyzed with PLS. The diet explained 82 % of the variation. Within the dietary variables, the percentage of the energy intake from PUFA and 18:2n6 was most associated with variation of the Bacteroides cluster (proportion of variation ~30 %), followed by energy from 18:3n3 (Fig. 7.2). WG and refined breads also had >10 % of their variation associated with Bacteroides cluster variation.

![Image 7.2](image_url)

*Figure 7.2.* Variation in nutrient and food intakes associated with the relative abundance of the Bacteroides cluster in bootstrap aggregated PLS analysis. Only nutrients and food groups with ≥10 % of associated variation are shown. E %, percentage of total energy intake; PLS, partial least square; WG, wholegrain.

When studying the overall associations between the microbiota composition and dietary intake for the entire data set, a total of 37 % of the variation in diet was associated with the microbiota composition, with the butyrate-producing *Faecalibacterium prausnitzii* having clearly the highest impact (PLS explained 72 % of the variation of *F. prausnitzii*), followed
by another butyrate producer, *Eubacterium rectale* (Supplemental Fig. 5). Among the 15 bacterial genera that were the most associated with diet, 7 are capable of producing butyrate from sugar but not from lactate (*F. prausnitzii, E. rectale, Lachnospira pectinoschiza, Roseburia intestinalis, E. cylindroides, Lachnobacterium bovis*, and *E. ventriosum*). A total of 97 % of the variation in these butyrate producers was explained by diet, so the intake of WG breads, refined white breads, total fiber, and grain fiber explained their variation the most (40–52 %) (Fig. 7.3).

![Proportion of variation](image)

*Figure 7.3.* Variation in nutrient and food group intakes associated with the relative abundance of butyrate producers in bootstrap aggregated PLS analysis. Only nutrients and food groups with ≥ 10 % of associated variation are shown. E %, percentage of total energy intake; PLS, partial least square; WG, wholegrain.

To explore the diet-microbiota associations identified in the PLS analysis, we analyzed bivariate mid-correlations between the abundance of each genus-like bacterial group and nutrient and food intakes. None of the detected correlations reached *r* = 0.5 or the default threshold for significance (P < 0.05). When using the looser threshold (P < 0.2) for exploratory purposes, the following weak correlations were observed: *B. vulgatus et rel.*, *B. ovatus et rel.*, *P. tannerae et rel.*, and *P. oralis et rel.* correlated (*r* ≥ 0.30) with the intake of 18:2n6, other fat-derived compounds, and margarine. Of the butyrate producers derived from the PLS analysis, *F. prausnitzii et rel.* correlated inversely with the intake of refined white breads (*r* = −0.35), and *R. intestinalis et rel.* correlated positively with that of total fiber (*r* = 0.33), grain fiber (*r* = 0.34), and WG breads (*r* = 0.35) and negatively with that of refined white breads (*r* = −0.30). The other butyrate producers, when analyzed individually, did not
correlate with any nutrients or foods, except for *E. ventriosum*, which correlated with energy intake from alcohol (r = 0.33).

### 7.5 DISCUSSION

In this 12-wk, randomized intervention study in participants with metabolic syndrome, we investigated if replacing the habitual intake of WG rye bread with refined WWB would affect intestinal microbiota composition. To our knowledge, this study is the first to couple community-level analysis of the intestinal microbiota to accurate dietary records and controlled change in the intake of grain products. Furthermore, this study addressed the impact of staple foods on the intestinal microbiota composition as opposed to added grain fiber fractions or fiber supplements previously analyzed (215,230).

The major difference between the intervention diets was the type of bread as well as the amount of whole grains consumed. The participants in both groups consumed nearly the same daily amount of grain products but of notably different quality. In the RB group, a total of 187 g/d of grain products was consumed, of which 92 g was WG breads and 60 g was high-fiber endosperm rye bread. The RB intervention diet included 75 g/d of whole grains, which is 1.5 times the amount recommended by U.S. dietary guidelines (12). In contrast, in the WWB group, 226 g/d of grain products was consumed, of which 188 g was refined white breads. Despite the substantial difference in the whole grain intake, which was confirmed by the difference in plasma AR concentration, the microbiota composition did not significantly differ between the groups. Similarly to our comprehensive microbiota analysis, no effect on the dominant intestinal bacteria was observed in a strictly controlled, 2-wk intervention study with 151 g/d of whole grains, consisting mainly of wheat, compared with refined grains (96). Instead, the addition of 60 g/d of WG barley or brown rice to a diet for 4 wk increased and decreased the abundance of Firmicutes and *Bacteroides*, respectively, in healthy American individuals (146). Unfortunately, the participants’ habitual diet was not described. Our intervention cannot be directly compared with other grain intervention studies in which participants’ baseline diet is composed of refined grain products. Based on our baseline observations and a national survey (231), Finns consume a relatively high amount of WG rye bread (on average 86 g/d). Long-term diets seem to be associated with compositional differences of the microbiota (216-218). Hence, the high, long-term consumption of whole grains might have affected our study population’s microbiota and its responsiveness to changes in the WG content of the diet. Furthermore, preliminary data show that individuals with metabolic syndrome differ in their microbiota composition from healthy individuals (130). Thus, the generalization of our results to populations with low WG intake or individuals without metabolic syndrome has to be made with caution.

Only the abundance of *Bryantella formatexigans* of the 1033 phylotypes detected by the HITChip was found to be significantly different with the WWB diet compared with the RB diet. *B. formatexigans*, belonging to the *Clostridium* cluster XIVa, requires carbohydrates and formate for growth and is able to ferment cellulose into acetate (232). Within-group microbiota changes were observed in only the WWB group, in which the participants
substituted refined white wheat bread for rye bread, whereas in the RB group only minor changes in the diet occurred. Thus, the habitual high-WG food consumption might explain the lack of change in the microbiota composition within the RB group.

Within the WWB group, a significant microbiota change occurred even at the phylum level. The change mostly manifested within a specific Bacteroides cluster in which a subset of phylotypes was affected (Supplemental Table 3), possibly reflecting variable metabolic or competitive properties within a group. Although significant, the magnitude of the changes in the bacterial abundance was modest (from a 19% decrease to a 17% increase) and specific to the individual, which is in line with previous observations (143,217). Of the significantly altered genera in the WWB group, B. vulgatus decreased the most. B. vulgatus can utilize rye arabinoxylan in vitro (233). Thus, removal of rye bread from the diet could explain the observed decrease in B. vulgatus, as many Bacteroides and Prevotella spp. have conserved and well-described molecular machineries to degrade and ferment a great variety of indigestible polysaccharides such as xylan (234,235), a component of arabinoxylan. Comparisons of the microbiota in westernized (Italy and US) and nonwesternized (rural Africa and Venezuela) countries have revealed the latter to be enriched with Prevotella spp., probably due to long exposure to diets rich in plant-derived, complex carbohydrates (216,218). However, in our study, there was a trend for decreased abundance of B. vulgatus also among the participants consuming rye bread. Accordingly, in multivariate regression analysis, the main diet-dependent explanatory factor for the abundance of the Bacteroides cluster was not the intake of WG breads or grain fiber, but that of fat-derived compounds. Their intake alongside the corresponding food items, such as spreads, margarine, and fish, varied largely among the individuals independent of the group. Although most of the ingested fat is absorbed in the small intestine, dietary fat might affect the colonic microbiota via modulation of bile acids (236). Previously, intake of fat was observed to be positively associated with Bacteroides-dominated microbiota and Enterotype 1 when analyzing long-term habitual diets (217) and negatively with the butyryl-CoA synthetase gene involved in butyrate production as well as butyrate producers (237). However, an intervention study showed no effect of SFA or MUFA on numbers of Bacteroides and Prevotella after 6 mo (238). In mice, a series of studies has shown that high-fat feeding affects the intestinal microbiota (132). Recently, the quality of ingested fat was also shown to influence the cecal microbiota via altered bile acid composition (239). Hence, several recent studies suggest that fat is a potential factor affecting the composition of the intestinal microbiota.

Certain members of Clostridium clusters IV and XI were slightly increased in the WWB group during the intervention. The Clostridial clusters IV and XIVa contain the main carbohydrate-utilizing butyrate producers in the human gut, with F. prausnitzii, R. intestinalis, and E. rectale being the most abundant (137). The abundance of these groups did not differ between the diets. Both rye and white wheat bread contain resistant starch (240,241), which may have affected the microbiota parallel to the quantified nondigestible carbohydrates. Recently, a positive association between butyrate-producing bacteria and insulin sensitivity was observed in participants with metabolic syndrome (131). Furthermore, an increase in the abundance of E. rectale was associated with improvement in postprandial glucose and insulin responses (146). Although the abundance of the main butyrate producers and state of glucose metabolism (86) remained the same during our
intervention, increased insulin sensitivity has been observed after daily intake of insoluble cereal fiber for 6 wk (242) without changes in dominant groups of intestinal microbiota (147). This may suggest that altered microbiota composition can contribute but is not necessary to improve insulin resistance.

Although diet altogether explained the majority of the variation in the Bacteroides cluster and certain butyrate producers in the PLS, any single nutrient or food group did not strongly correlate with the individual implicated bacterial groups. However, the observed correlations, although weak, were mainly in line with those derived from the PLS analysis. Our results suggest that multivariate analyses constitute a biologically more informative approach than analyses of each taxa separately, because they also capture subtle differences. Moreover, different members of the microbiota do not operate in isolation but as part of the community that is known to possess a high rate of functional redundancy and cross-feeding among the species. For example, the utilization of complex grain polysaccharides consisting of nonsoluble and soluble particles is carried out by a concerted action of different primary and secondary degraders (137). In this study, the detected high variation of Bifidobacteria was not associated with intervention group or any dietary variable, even in PLS analysis. The intake of oligosaccharide- or other prebiotic-containing foods (230) was, unfortunately, not controlled and may have contributed to the variation.

In conclusion, a high compared with low intake of whole grains for 12 wk did not differentiate the intestinal microbiota composition in participants with metabolic syndrome. However, across the entire cohort, we identified changes in the microbiota composition that were associated mostly with the intake of fat-derived compounds and to a lesser extent with that of WG foods. Our results highlight the fact that intentional modulation of the microbiota by withdrawal or supplementation of carbohydrate-containing staple foods is not straightforward, because the baseline microbiota as well as intake of minor dietary components, such as fatty acids, contributes to the outcome. To clarify the effect of diet on the intestinal microbiota composition, different types and sources of dietary fiber as well as the amount and quality of fat should be carefully controlled in further intervention studies.
8 Comparison of postprandial phenolic acid excretions and glucose responses after ingestion of breads with bioprocessed or native rye bran\textsuperscript{4} (Study IV)

8.1 ABSTRACT

Rye bran contains a high amount of phenolic acids with potential health promoting effects. However, due to binding to dietary fibre, the phenolic acids are poorly absorbed in human body. We used bioprocessing with enzymes and yeast to release phenolic acids from the fibre complex and studied the effect of bioprocessing on absorption of phenolic acids in healthy humans. White wheat breads fortified with bioprocessed or native rye bran, and wholegrain rye bread and white wheat bread as controls were served to 15 subjects in randomized order in cross-over design. Urine was collected at basal state and over 24 hours in four-, eight-, and twelve-hour periods and analyzed for phenolic acids and their metabolites with gas chromatography. A total of six blood samples were taken over four hours to study the effect of the bread ingestion on postprandial glucose and insulin responses. Bioprocessing of rye bran increased the proportion of free ferulic acid (FA) and soluble arabinoyxlan in the bread. Ingestion of the white wheat bread fortified with bioprocessed rye bran increased (p<0.001) urinary excretion of FA particularly during the first four hours, indicating increased absorption of FA from the small intestine. The postprandial glucose and insulin responses were similar between these breads. Bioprocessing of rye bran did not affect excretion of benzoic, phenylpropionic, and phenylacetic acid metabolites. As a conclusion, bioprocessed rye bran as compared with native rye bran increased absorption of FA from the small intestine, but did not improve postprandial glucose and insulin responses.

8.2 INTRODUCTION

Epidemiological studies consistently show a decreased risk of type 2 diabetes and cardiovascular diseases due to consumption of wholegrain foods (17,18). However, mechanisms explaining the reduced risk are unknown. Among the suggested mechanisms, the intake of antioxidative compounds associated with the fibre complex of grains and the beneficial effects on postprandial glucose and insulin responses presumably play a role in the decreased risk of diseases (7,243).

The most abundant phenolic compounds, in quantity, in rye grain and rye and wheat products are phenolic acids, particularly ferulic acid (FA) followed by sinapic and p-

coumaric acids (66,67). The highest content of phenolic acids is found in the bran (66). The health promoting effects of grain phenolic acids have been mainly studied in vitro and ex vivo regarding their antioxidative and anti-inflammatory capacity (153,243,244). In the human body, phenolic acids should be released and absorbed before they can be expected to affect cells and tissues other than in the intestinal tract. The majority of FA is ester-linked to arabinoxylan (AX), forming large molecules which cannot be absorbed across the enterocytes. In rye, less than 4% of the total phenolic acids exist in a free form (55), and thus being available for absorption from the small intestine.

A few studies in humans have shown that some FA (most probably the free form) in wheat bran is absorbed from the small intestine 1-3 hours after ingestion of a single meal (152-154). Urinary excretion reflected well the absorption since the peak of urinary phenolic acid concentration was reached two hours after ingestion of wheat bran whereas in plasma the peak concentration was reached after one hour (154). However, there are no human data about the postprandial absorption of phenolic acids in rye bran, although significant absorption of FA was shown to take place over six-week daily consumption (155). In addition, a 2-fold higher concentration of phenolic acid metabolites was detected in urine after ingestion of 48 g wheat bran or whole grain breakfast cereals daily for three weeks as compared with no bran or whole grain cereals in diet (142).

Harder et al. (155) also showed that only 28% of FA in the consumed rye bran was absorbed and excreted in urine as free or conjugated forms. This suggests that conversion to other phenolic metabolites is involved prior to urinary excretion. Indeed, the proportion of FA bound to AX is not absorbed but moved along the large intestine (158). In the large intestine, microbiota esterases release FA, sinapic, and p-coumaric acids from the fibre complex (157). FA, along with other phenolic acids, is metabolized by the microbiota to propionic, acetic, and benzoic acid derivatives (159-161), which are absorbed from the large intestine (153,162). The continuous flow of colonic metabolites into the circulation due to regular consumption of wholegrain foods has been suggested to contribute to the health benefits of whole grains (53).

Bioprocessing combining fermentation and enzymatic treatment increases the amount of free phenolic acids in wheat bran (159) and increases their absorption from the intestine (153). Bioprocessing by fermentation or enzyme technology also solubilizes AX in rye bran (245,246). When cell wall polysaccharides are partially hydrolysed due to bioprocessing, enhanced large intestinal fermentation and production of phenolic metabolites is hypothesized to occur (159). Furthermore, soluble AX and free phenolic acids in bread have been shown or suggested to beneficially affect postprandial glucose and insulin responses (108,189).

In this study, we aimed firstly to investigate the extent of absorption of phenolic acids and their metabolites for 24 hour after ingestion of breads enriched with native rye bran or rye bran bioprocessed with enzymes and yeast, secondly the time course of absorption, i.e. whether the phenolic acids and metabolites were absorbed in the initial 4-hour postprandial period or later, and thirdly whether the bioprocessed rye bran baked in white wheat bread improved the 4 hour postprandial glucose and insulin responses.
8.3 MATERIALS AND METHODS

8.3.1 Subjects
Fifteen healthy subjects (nine females, six men) fulfilling inclusion criteria started and finished an extended postprandial study. The subjects were between 35 and 65 years (mean 57 y) and had a BMI between 21 and 32 kg/m² (mean 26 kg/m²). The inclusion criterion for the study was mild, moderate, or severe self-reported gastrointestinal symptoms after ingestion of cereal foods, particularly rye bread. This inclusion criterion was used for other aims of the study not reported in this manuscript. Exclusion criteria included celiac disease, cereal or milk protein allergy, vegetarian diet, other special diet (such as low-carbohydrate diet), inflammatory bowel disease, other chronic disease (hypertension and hyper- or hypothyroidism controlled with medication were allowed), antibiotic use over the preceding two months, and blood donation over the preceding three months. The subjects provided written informed consent to participate in the study. The study was approved by the Ethics Committee of the Hospital District of Northern Savo.

8.3.2 Test breads
Breads included in the study were a commercial wholegrain rye bread (R bread; conventional rye bread with 100 % rye flour; Vaasan Oy, Finland), a white wheat bread (WW bread), a white wheat bread fortified with native rye bran (RB+WW bread) and a white wheat bread fortified with bioprocessed rye bran (BRB+WW bread). Rye bran was provided by Fazer Group (Lahti, Finland). Prior to bioprocessing and baking, the bran was milled with a pin disc (17 800 rpm) of Alpine Fine Impact Mill 100 UPZ-Ib (Hosokawa Alpine AG, Germany) and air-classified (rotor speed 3000 rpm) with Minisplit Classifier (British Rema Manufacturing Company Ltd., UK) to reduce the starch content from 40 % to 15.7 %. For bioprocessing, the bran was first treated with a hydrolytic enzyme mixture, with cell-wall degrading properties, of Depol 740L (Biocatalysts; dosing 200 nkat/g bran, based on xylanase activity) containing ferulic acid esterase activity and Grindamyl A 1000 (dosing 75 nkat/g bran, based on amylase activity) at 65 % water content in 40 °C for four hours, with mixing at 0.5, 1, 2, 3, and 4 hours. Thereafter, Baker’s yeast (1.25 %) (Sunnuntai) was added and the mixture was fermented at 20 °C for 20 hours. For preparing the BRB+WW and RB+WW breads, 35 % of the white wheat flour (Leipurin vehnäjauhu V600, Fazer) was replaced with the bran samples (BRB and RB), based on dry matter. In addition, the BRB+WW, RB+WW and WW bread doughs contained (of flour weight): yeast (Sunnuntai; 4.4-5 %), sugar (1.9-2.0 %), salt (1.4-1.5 %), baking margarine (Sunnuntai, Raisio; 11.3-12.0 %), and emulsifier (Panodan; 0.5 %) (the smaller values are for BRB+WW). The doughs were left to rest for 20 min in 28 °C and 75 % relative humidity, mixing twice for two and four minutes during resting. The breads were prepared in 400 g dough pieces, proofed for 50 min at 35 °C and 80 % relative humidity, and baked at 225 °C for 20 min, with 15 seconds of steaming in the beginning. For analysis of the chemical composition, the breads were freeze-dried, and the dietary fibre, beta-glucan, AX, fructan, protein, fat, starch, and phenolic acid contents of the breads were determined as described by Nordlund et al. (160).

8.3.3 Study design
The study followed a randomized, cross-over design, the subjects serving as their own controls. Known confounding factors were standardized by advising the subjects to
maintain their body weight and lifestyle habits throughout the study. The subjects were asked to avoid unusually large portions of food on the day preceding each test, and avoid consumption of alcohol for two days before each study visit. On the first study visit, the subjects were asked to report their previous evening meal and to consume as similar evening meal as possible preceding every forthcoming study visit. One-day food records describing the diet preceding each study visit were filled in by the subjects and analyzed using Diet32 software (version 1.4.6.3, Aivo Finland Oy, Turku, Finland) for nutrient intakes. Furthermore, the habitual intake of coffee, tea, and grain products containing wholegrain wheat and rye was examined from the records because they provide the most of the phenolic acids in Finnish diet (247).

Urine receivers were provided for the subjects for collection of excreted urine for 12 hours prior to ingestion of test meals (Fig. 8.1). After a 12-hour overnight fasting, the body weight of the subjects was measured and an intravenous catheter was inserted in the antecubital vein of the arm. Fasting blood samples were taken before starting ingestion of the test meal.

The test meals were served to each subject in random order with at least three days between each test meal. The test meal included one of the test breads providing 50 g of available starch. In addition, the test meal included 40 g cucumber, 20 g milk-free margarine (Keiju 60 %, Raisio), and 3 dl water, or 1.75 dl filter coffee or black tea and 1.25 dl water if the subject was used to drink coffee/tea in the morning. If the subject drank coffee/tea on the first study visit, the same drink was served on each of the following occasions to keep the meal composition constant. Meal eating time was restricted to 15 minutes. Five blood samples were taken after commencing of eating the test meal (at 30, 60, 120, 180, and 240 min) for measurements of plasma glucose and insulin concentrations (Fig. 8.1).

Figure 8.1. Study design.

After the four-hour postprandial phase, a standardized lunch was served to the subjects. The lunch included a slice of the same test bread served on the morning test meal so that the amount of fibre from the breakfast and lunch was altogether 20 g for the BRB+WW, RB+WW, and R breads, and 4.8 g for the WW bread. Ingestion of the test bread was compulsory but the amount of other components of the lunch voluntary. The other components were chicken soup, lettuce, tomato, grated carrot, salad dressing, milk-free
margarine (Keiju 60 %, Raisio), lactose-free milk or sour milk, artificially sweetened juice, and water. During the rest of the day the subjects were allowed to eat without restriction but following their normal dietary habits.

Urine was collected for four hours after ingestion of the test meal as a 0-4 hour sample, for eight hours as a 4-12 hour sample, and for twelve hours as a 12-24 hour sample. The subjects returned the urine receivers on the following morning to the study laboratory. Total volume of excreted urine was recorded for each time period.

8.3.4 Urine samples
After adding formic acid to the urine (100 μl per 5 ml urine), the samples were stored in a freezer at -80 °C for analyses of phenolic acids and their metabolites.

Prior to analysis of phenolic acids 500 μl urine was incubated in 1500 μl acetic acid buffer (0.15M, pH 4.1) containing 0.10 mg /ml Helix pomatia enzyme mixture (Sigma G071-500 KU) overnight at 37 °C to hydrolyze conjugated metabolites. The method therefore describes the sum of non-conjugated (i.e. free) and originally glucuronidated phenolic acids.

After the hydrolysis the 2 ml sample was purified using OASIS HLB cartridges and eluted with 1 ml methanol. An aliquot of 400 μl methanol extract was drawn, evaporated to dryness and silylated using 25 μl MOX (45 °C, 1 h) and 26 μl MSTFA (45 °C, 1 h). Analysis was performed by gas chromatography as described in Aura et al (248), including FA and sinapic acid standards. Reagents for the targeted analysis of metabolites of phenolic acids using gas chromatography with mass spectrometry (GC-MS) were as follows: 2-hydroxycinnamic acid (Aldrich, Steinheim, Germany; CAS 614-60-8) was used as the internal standard. The following compounds were used as external standards: benzoic acid (CAS 65-85-0), 3-hydroxybenzoic acid (CAS 99-06-9), 3,4-dimethoxybenzoic acid (CAS 93-07-2), 3-(4'-hydroxyphenyl)propionic acid (CAS 501-97-3) and 3-(3',4'-dihydroxyphenyl) propionic acid (CAS 1078-61-1) were purchased from Aldrich, (Steinheim, Germany); 4-hydroxybenzoic acid (CAS 99-96-7), 2-(3'-hydroxyphenyl)acetic acid (CAS 621-37-4) and 2-(3',4'-dihydroxyphenyl)acetic acid (CAS 102-32-9) were purchased from Sigma (St. Louis, USA); 3-phenylpropionic acid (CAS 501-52-0) and 3,4-dihydroxybenzoic acid (CAS 99-50-3) were from Fluka (Buchs, Switzerland); and 3-(3'-hydroxyphenyl)propionic acid (CAS 621-54-5) was purchased from Alfa Aesar (Karlsruhe, Germany). Methoxyamine hydrochloride (2%) in pyridine (MOX; Pierce, Rockford, USA) and N-Methyl-N-trimethylsilyl-trifluoracetamide (MSTFA) from Pierce (Rockford, USA) was used as the derivatization reagent.

8.3.5 Biochemical analyses
Plasma glucose was analyzed using the glucose dehydrogenase photometric method (Konelab System Reagents and KoneLab 20XTi Clinical Chemistry Analyser, Thermo Fisher Scientific, Vantaa, Finland). Plasma insulin was analyzed with a chemiluminescent immunoassay (Advia Centaur Immunoassay System, Siemens Medical Solution Diagnostics, Tarrytown, NY, USA).
8.3.6 Calculations
Urine was not fully recovered from one subject during the R, RB+WW, and BRB+WW bread meal periods; thus, the data regarding urinary excretions are from fourteen subjects during these periods and from fifteen subjects during the WW bread meal period.

The urinary excretion of phenolic compounds was grouped into benzoic, phenylpropionic, and phenylacetic acid metabolites and reported as the sum of the individual metabolites. The benzoic acid metabolites were vanillic acid, benzoic acid, 3,4-dimetoxybenzoic acid, 3,4-dihydroxybenzoic acid, 3-hydroxybenzoic acid, and 4-hydroxybenzoic acid. The phenylpropionic acid metabolites were 3-(3’, 4’-dihydroxyphenyl)propionic acid, 3-(3’-hydroxyphenyl)propionic acid, 3-(4’-hydroxyphenyl)propionic acid, and 3-phenylpropionic acid. The phenylacetic acid metabolites were 2-(3’-hydroxyphenyl)acetic acid and 2-(3’, 4’-dihydroxyphenyl)acetic acid.

Total 24-hour excretion of phenolic acids and metabolites after ingestion of the bread meals is reported as sum of the 0-4, 4-12, and 12-24 hour excretions. To compare changes in the excreted amounts of phenolic acids and the metabolites among the bread meals, the 12-hour basal excretion on each occasion was subtracted from the total 24-hour excretion.

The course of glycemia to describe form of the glucose curve was analyzed by calculating the glycemic profile (GP) (100): time (min) during which blood glucose concentration was above the fasting concentration was divided with the incremental peak value (mmol/l) of blood glucose for each subject and test meal. The incremental area under the curve (iAUC) of glucose and insulin was calculated from the area beneath the curve above the fasting concentration including only the area before the concentration dropped below the fasting level. For GP and iAUC calculations, GraphPad Prism 4.0 for Windows (GraphPad Software, Inc., San Diego, CA) was used.

8.3.7 Statistics
To compare changes in the excreted amounts of phenolic acids and metabolites among the bread meals, the nutrient and food intakes preceding each test occasion, and GP among the bread meals, the General linear model (GLM) for repeated measures was used for normally distributed variables and Friedman’s test followed by the Wilcoxon singed rank test was used for non-normally distributed variables. The p-values were adjusted for multiple comparisons (Bonferroni).

Linear mixed effect modeling was used to compare the effects of the test meals on excretion of phenolic acids at individual time periods and on postprandial glucose and insulin responses. Histograms were used for checking the normality of model residuals, and logarithmic transformation was used for non-normally distributed data. The statistical significance was tested in the mixed-model analysis by using the subject as a random factor and time x meal and their main effects as fixed factors. For differences among the bread meals at individual time points, the fasting values were used as covariates only when the fasting values differed among the meals. Differences between the meals at individual time points were tested by post hoc analysis with adjustment for multiple comparisons (Bonferroni).
Correlation between the total intake of free FA from the test breads and the total 24-hour urinary excretion of FA was performed using Spearman’s correlation.

Statistical analyses were conducted with SPSS 19.0 for Windows (Chicago, IL). p-values < 0.05 were regarded statistically significant. Data is expressed as mean or mean ± SD.

8.4 RESULTS

8.4.1 Test breads
The chemical composition of the test bread portions at breakfast is reported in Table 8.1. The bread portions varied from 109 to 166 g to give the same amount of available starch for studying the postprandial glucose and insulin responses. The total fibre content of the meal ranged from 3.8 g to 19.1 g among the breads. The BRB+WW bread portion contained the highest amount of soluble AX (3.8 g, comprising 46 % of the total AX content) and total phenolic acids. Particularly, the free FA content was clearly the highest in the BRB+WW in which 20 % of FA was in the free form, whereas in the RB+WW bread the figure was only 2 %. The WW bread contained the least fibre (3.8 g) and total FA (6.6 mg).

8.4.2 Diet
Based on the food record analysis, the intake of nutrients was the same preceding each test day (data not shown). Furthermore, subjects’ intake of wholegrain wheat and rye containing products, coffee, and tea did not differ among the days preceding the test day (mean intakes of the four occasions for rye breads 21 ± 35 g per day, rye containing pastries 22 ± 51 g per day, wholegrain wheat breads 26 ± 41 g per day, other wholegrain wheat containing products 31 ± 81 g per day, for coffee 3.6 ± 2.6 dl per day, and for tea 1.2 ± 1.8 dl per day). At each test meal, eleven out of fifteen subjects drank coffee, and three subjects drank tea. The weight of the subjects remained the same during the whole study (data not shown).

8.4.3 Urinary excretion of phenolic acids and metabolites
Increments from the basal excreted amounts of FA and sinapic acid differed among the test meals over 24 hours (p<0.001) (Fig. 8.2 A, B). After ingestion of the BRB+WW bread meal, the increment in FA excretion was 4-fold greater as compared to that of the RB+WW bread meal (p<0.001), and 8-fold greater as compared to that of the R and WW bread meals (p<0.001) (Fig. 8.2 A). Furthermore, after ingestion of the RB+WW bread meal, the increment in FA excretion was almost 2-fold greater as compared to that of the R bread (p<0.05) and the WW bread meals (p<0.01).

The amount of excreted sinapic acid was clearly less than that of FA in general (Fig. 8.2 A, B). After ingestion of the BRB+WW bread meal, excretion of sinapic acid was slightly greater than after that of the RB+WW bread meal (p<0.05) (Fig. 8.2 B). The total amount of and the increment in 4-coumaric acid excretion were very small and did not differ among the test meals (data not shown). Increments in excreted amounts of benzoic, phenylpropionic, and phenylacetic acid metabolites were the same among the test meals (Figure 8.2 C-E).
Table 8.1. Nutrient content of the ingested bread portions\(^a\) at breakfast

<table>
<thead>
<tr>
<th>Portion (g)</th>
<th>Protein (g)</th>
<th>Fat (g)</th>
<th>Total fibre (g)</th>
<th>Total AX (g)</th>
<th>Soluble AX (g)</th>
<th>Fructan (g)</th>
<th>β-glucan (g)</th>
<th>FA (mg)</th>
<th>Sinapic acid (mg)</th>
<th>4-coumaric acid (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Total</td>
<td>Free</td>
</tr>
<tr>
<td>BRB+WW</td>
<td>166</td>
<td>15.8</td>
<td>10.1</td>
<td>16.8</td>
<td>8.3</td>
<td>3.8</td>
<td>1.2</td>
<td>0.8</td>
<td>134.6</td>
<td>27.4</td>
</tr>
<tr>
<td>RB+WW</td>
<td>164</td>
<td>14.5</td>
<td>9.7</td>
<td>19.1</td>
<td>7.6</td>
<td>1.5</td>
<td>2.0</td>
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<td>117.0</td>
<td>2.2</td>
</tr>
<tr>
<td>R</td>
<td>123</td>
<td>9.2</td>
<td>1.0</td>
<td>16.4</td>
<td>5.3</td>
<td>1.7</td>
<td>2.4</td>
<td>1.7</td>
<td>74.0</td>
<td>0.5</td>
</tr>
<tr>
<td>WW</td>
<td>109</td>
<td>9.6</td>
<td>6.1</td>
<td>3.8</td>
<td>1.3</td>
<td>0.8</td>
<td>0.4</td>
<td>0.2</td>
<td>6.6</td>
<td>0.2</td>
</tr>
</tbody>
</table>

\(^a\)Each test bread portion contained 50 g of available starch. AX, arabinxylan; BRB+WW, white wheat bread fortified with bioprocessed rye bran; FA, ferulic acid; R, rye bread; RB+WW, white wheat bread fortified with native rye bran; WW, white wheat bread.
Figure 8.2. Amounts of FA (A) and sinapic (B) acid, and benzoic (C), phenylpropionate (D), and phenylacetic (E) acid metabolites excreted in urine 24 hours after ingestion of the bread meals. The incremental values (i.e., change from the basal excretion) are shown. Different from BRB+WW: ***p<0.001, **p<0.01, *p<0.05. Different from RB+WW: ##p<0.05, #p<0.05. BRB+WW, white wheat bread fortified with bioprocessed rye bran; R, rye bread; RB+WW, white wheat bread fortified with native rye bran; WW, white wheat bread. Data represents mean ± SD for 15 subjects regarding WW bread meal period and for 14 subjects regarding the other periods.

When comparing urinary excretions at individual time periods, we gained information about the time course of excretion of FA and the metabolites after ingestion of the bread meals. The excreted amount of FA for four hours (0-4 h) and the following eight hours (4-12 h) was greater after the ingestion of the BRB+WW bread meal than after that of the RB+WW bread meal (p<0.001) and the other bread meals (p<0.001) (Table 8.2). Most of the FA excretion occurred during four hours after ingestion of the BRB+WW bread meal. For the
following twelve hours (12-24 h), the excretion was greater only when the BRB+WW bread was compared to the R bread and WW bread meals (p<0.01). Also ingestion of the RB+WW bread meal resulted in greater excretion of FA for four hours (0-4 h) as compared to that of the R and WW bread meals (p<0.001). The excreted amount of sinapic acid did not differ between the BRB+WW and RB+WW bread meals at any individual time period, although there were differences in sinapic acid excretion between the other bread meals (Table 8.2). The time courses of excretion of benzoic, phenylpropionic, and phenylacetic metabolites were similar between the BRB+WW and RB+WW bread meals (time x meal interaction p>0.05; no difference at individual time periods).

Table 8.2. Urinary excretion of FA and sinapic acid over different time periods after ingestion of the bread test meals

<table>
<thead>
<tr>
<th></th>
<th>BRB+WW</th>
<th>RB+WW</th>
<th>R</th>
<th>WW</th>
<th>P-value*a</th>
</tr>
</thead>
<tbody>
<tr>
<td>FA, μmol</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>-12 to 0 h</td>
<td>3.0 ± 1.8</td>
<td>2.1 ± 1.3</td>
<td>3.1 ± 1.7</td>
<td>2.2 ± 1.1</td>
<td></td>
</tr>
<tr>
<td>0-4 h</td>
<td>25.2 ± 10.9</td>
<td>4.9 ± 2.1***</td>
<td>2.6 ± 1.6***,†††</td>
<td>1.6 ± 0.8***,†††</td>
<td></td>
</tr>
<tr>
<td>4-12 h</td>
<td>12.0 ± 6.9</td>
<td>4.3 ± 2.0***</td>
<td>3.8 ± 1.9***</td>
<td>3.1 ± 1.4***</td>
<td></td>
</tr>
<tr>
<td>12-24 h</td>
<td>5.5 ± 5.2</td>
<td>2.6 ± 1.3</td>
<td>2.1 ± 1.4**</td>
<td>2.2 ± 1.2**</td>
<td></td>
</tr>
<tr>
<td>Sinapic acid, μmol</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>-12 to 0 h</td>
<td>1.2 ± 1.3</td>
<td>0.4 ± 0.4</td>
<td>0.8 ± 0.6</td>
<td>0.7 ± 0.8</td>
<td></td>
</tr>
<tr>
<td>0-4 h</td>
<td>2.2 ± 0.8</td>
<td>0.9 ± 0.4</td>
<td>0.3 ± 0.2***,†††</td>
<td>0.1 ± 0.0***,†††,#</td>
<td></td>
</tr>
<tr>
<td>4-12 h</td>
<td>2.0 ± 1.2</td>
<td>1.1 ± 0.9</td>
<td>0.9 ± 1.0**</td>
<td>0.8 ± 0.5**</td>
<td></td>
</tr>
<tr>
<td>12-24 h</td>
<td>1.0 ± 0.8</td>
<td>0.8 ± 0.7</td>
<td>0.7 ± 1.3*,†</td>
<td>0.5 ± 0.4</td>
<td></td>
</tr>
</tbody>
</table>

*a P-value for time x meal interaction. Different from BRB+WW: ***p<0.001, **p<0.01, *p<0.05. Different from RB+WW: †††p<0.001, †p<0.05. Different from R: ##p<0.01. BRB+WW, white wheat bread fortified with bioprocessed rye bran; FA, ferulic acid; RB+WW, white wheat bread fortified with native rye bran; WW, white wheat bread; R, rye bread. Data represents mean ± SD for 15 subjects regarding WW bread meal period and for 14 subjects regarding the other periods.

Only approximately 1% of the ingested total FA and sinapic acid in the breads containing rye was detected as FA and sinapic acid in the excreted urine after ingestion of the meals (Table 8.3). The total excretion of FA in urine as a percent of the intake was 2.5-fold after ingestion of the BRB+WW bread than after that of the RB+WW bread (Table 8.3). Ingestion of the RB+WW bread and R bread caused similar urinary excretion of FA and sinapic acid relative to their intakes.

The intake of free FA from the test breads correlated positively with the total 24-hour urinary excretion of FA (r=0.77, p<0.01).
Table 8.3. Total intake of phenolic acids from the breads ingested at breakfast and lunch, and absolute and percentual phenolic acid excretion during 24 hours\(^a\)

<table>
<thead>
<tr>
<th></th>
<th>BRB+WW</th>
<th>RB+WW</th>
<th>R</th>
<th>WW</th>
</tr>
</thead>
<tbody>
<tr>
<td>FA intake, mg</td>
<td>160.6</td>
<td>122.7</td>
<td>91.5</td>
<td>8.4</td>
</tr>
<tr>
<td>Excreted amount in urine, mg (%)</td>
<td>1.66 ± 0.51 (1.0)</td>
<td>0.45 ± 0.15 (0.4)</td>
<td>0.33 ± 0.14 (0.4)</td>
<td>0.27 ± 0.10 (3.2)</td>
</tr>
<tr>
<td>Sinapic acid intake, mg</td>
<td>37.3</td>
<td>30.2</td>
<td>21.1</td>
<td>2.2</td>
</tr>
<tr>
<td>Excreted amount in urine, mg (%)</td>
<td>0.23 ± 0.09 (0.6)</td>
<td>0.12 ± 0.06 (0.4)</td>
<td>0.07 ± 0.06 (0.3)</td>
<td>0.06 ± 0.03 (2.8)</td>
</tr>
<tr>
<td>4-coumaric acid intake, mg</td>
<td>5.6</td>
<td>5.2</td>
<td>3.6</td>
<td>0.3</td>
</tr>
<tr>
<td>Excreted amount in urine, mg (%)</td>
<td>0.02 ± 0.01 (0.3)</td>
<td>0.02 ± 0.01 (0.3)</td>
<td>0.01 ± 0.01 (0.3)</td>
<td>0.01 ± 0.01 (3.8)</td>
</tr>
</tbody>
</table>

\(^a\) BRB+WW, white wheat bread fortified with bioprocessed rye bran; FA, ferulic acid; R, rye bread; RB+W, white wheat bread fortified with native rye bran; WW, white wheat bread. Data on urinary excretion represent mean ± SD for 15 subjects regarding WW bread meal period and for 14 subjects regarding the other periods.

8.4.4 Postprandial glucose and insulin responses

Postprandial glucose responses (Fig. 8.3 A) as well as GP and iAUC were similar for all the bread meals (data not shown). Postprandial insulin responses differed among the meals over four hours (p<0.01; time x meal interaction). At 60 min, plasma insulin concentration was significantly lower after ingestion of the R bread meal than that of the WW bread meal (p<0.05) (Fig. 8.3 B). The insulin iAUC differed among the meals (p<0.001) so that the iAUC for the R bread meal was significantly smaller than for the WW (p<0.001), RB+WW (p<0.01), and BRB+WW bread meals (p<0.05) (Fig. 8.3 C).
Figure 8.3. Plasma glucose (A) and insulin (B) responses and insulin iAUC (C) after ingestion of the bread meals. R different from WW: *p<0.05. Different from R: ***p<0.001, **p<0.01, *p<0.05. BRB+WW, white wheat bread fortified with bioprocessed rye bran; iAUC, incremental area under the curve; R, rye bread; RB+WW, white wheat bread fortified with native rye bran; WW, white wheat bread. Data represents mean ± SD for 15 subjects.

8.5 DISCUSSION

The phenolic acids bound to the dietary fibre complex of rye bran are poorly absorbed in the human body. In this study we showed that bioprocessing of rye bran with cell-wall and starch degrading enzymes and yeast significantly increased the absorption of liberated FA. On the other hand, hydrolysis of cell wall polysaccharides due to bioprocessing did not change the excretion of phenolic metabolites in urine.
Relative to the FA intake, clearly more FA was excreted in urine after ingestion of the bread fortified with bioprocessed than with native rye bran. Since the total ingested amount of BRB+WW bread provided only 1.3-fold more total FA than that of the RB+WW bread, the higher proportion of free FA in the BRB+WW bread explains the increased excretion. The result is presumably mainly due to the synergetic effect of xylanase and FA esterase during processing. This result is in line with our previous work with wheat bran, where bioprocessing with cell-wall degrading enzymes and yeast also increased the amount of free phenolic acids available for absorption (153). Ingestion of the RB+WW bread and R bread resulted in a similar urinary excretion of FA and sinapic acid as a percent of the intake, although the absolute amounts excreted were higher after the RB+WW bread because of the bigger portion size. Subjects in previous wheat bran studies had followed a low polyphenol diet excluding whole grain foods, coffee, and tea before and during the test day (152,153), which is in contrast to our study, where we wanted to study the effects of different breads as part of a habitual diet. The crossover design used in the present work enables comparison of FA excretion among the bread meals. However, the standardized portion of coffee contributed to the total excretion of FA. Ingestion of coffee rich in chlorogenic acid increases the concentration of plasma FA already during the first hour after the ingestion, although most of the chlorogenic acid is not metabolized until in the large intestine (249-252). Intestinal microbiota esterases hydrolyse chlorogenic acid and produce metabolites, such as phenylpropionic acid derivatives (253,254), also reported for black tea and FA (254-256).

Most of the ingested FA and sinapic acid was not excreted in urine either as free or conjugated during 24 hours suggesting the metabolism to other compounds, prolonged elimination due to enterohepatic circulation, or lack of absorption. However, the 24-hour metabolite excretions were similar after ingestion of all the rye bran-containing breads to that of the low fibre WW bread containing very little phenolic acids. Furthermore, degrading cell-wall polysaccharides of rye bran by bioprocessing did not contribute to enhanced excretion of metabolites in urine, in contrast to an in vitro study that showed bioprocessing of wheat bran to enhance formation of 3-phenylpropionic acid (159). However, bioprocessing of wheat bran did not increase 24-hour excretion of several phenolic metabolites in vivo, and the amount of some of the reported metabolites were actually decreased in the excreted urine as compared with native wheat bran (153).

Similar excretions of the benzoic, phenylpropionate, and phenylacetic acid metabolites among the test occasions are most probably explained by our subjects’ habitual diet containing foods rich in phenolic compounds which share the same metabolites, such as rye and wheat products, coffee, tea, berries, and vegetables (163). The basal FA excretion (estimated from the 12-hour excretion multiplied by two) was 1.6-fold higher in our study than in the study by Kern et al. (152) in which polyphenol rich foods had been excluded from subjects’ habitual diet before the test occasions. Furthermore, Costabile et al. (142) reported that the basal urinary concentration of 3,4-dihydroxyphenylpropionic acid and 4-hydroxybenzoic acid was 0.3 μmol/l for the both metabolites when the subjects avoided eating bran and wholegrain cereals. We instead observed several fold higher basal urinary concentrations of both acids (5.7 μmol/l and 2.9 μmol/l, respectively; means of the four
occasions). It is plausible that metabolites from the subjects’ habitual diet were circulating in the body and contributed to the observed excretions, while the bread with bioprocessed rye bran, and the other test breads, ingested within the 24 hours did not notably increase the metabolite excretions.

Ingestion of the BRB+WW bread did not improve the postprandial glucose and insulin responses as compared to that of the RB+WW bread, suggesting that free FA and solubilized AX in bread do not affect the postprandial responses. A previous study showed that a wheat bread enriched with both 12 g and 6 g of isolated AX (of which < 5.0 and < 2.5 g soluble, respectively) decreased postprandial glucose and insulin responses in healthy subjects (189). The BRB+WW bread portion contained 8.3 and 3.8 g of total and soluble AX, respectively, without decreasing the responses. The lack of effect might be partly explained by efficient enzyme treatment during the bioprocessing which probably reduced the molecular weight of AX to such an extent that the viscosity elevating properties of AX which are important for the reduced postprandial responses (257) were lost.

Characteristics of the subjects should be taken into account regarding the observed intestinal fermentation and excretion of phenolic metabolites. The subjects were recruited based on self-reported gastrointestinal symptoms which occur after ingestion of grain products. Individuals with irritable bowel syndrome are known to have alterations in their intestinal microbiota composition as compared to healthy persons (258). The composition of the intestinal microbiota most probably affects the conversion of phenolic acids in the large intestine (259). Although our subjects were not diagnosed with irritable bowel syndrome, their intestinal microbiota composition might be different from those who do not report suffering from intestinal symptoms after ingestion of grain products.

Although ingestion of bioprocessed rye bran as compared to native rye bran increased absorption and circulation of FA in the body it remains to be elucidated whether this phenomenon has actual health promoting effects. Ingestion of bioprocessed wheat bran was previously shown to exert anti-inflammatory effects ex vivo (153). However, the proportion of phenolic acids not released and absorbed may also have high antioxidant activity within the large intestine (243). It has also been suggested that the absorption of phenolic acid metabolites in the large intestine might contribute to health benefits of wholegrain foods (53). Our subjects seemed to have rather high concentration of phenolic acid metabolites circulating in the body due to habitual diets containing rye and wheat products, coffee and tea. Thus, it seems that the phenolic acid metabolites from such habitual diet make the amount produced by bioprocessing of bran less apparent.

### 8.6 CONCLUSIONS

As compared with native bran, rye bran bioprocessed with enzymes and yeast as a part of white wheat bread increased the absorption of FA. The increase was the highest within the first four hours after ingestion, suggesting that the small intestine was the main site of absorption. The increase in the absorption of FA from the small intestine is due to
conversion of bound FA into free FA. However, the postprandial glucose and insulin responses over four hours were not improved due to the bioprocessing of bran.
9 Postprandial glucose metabolism and SCFA after consuming wholegrain rye bread and wheat bread enriched with bioprocessed rye bran in individuals with mild gastrointestinal symptoms\textsuperscript{5} (Study V)

9.1 ABSTRACT

\textbf{Background:} Rye bread benefits glucose metabolism. It is unknown whether the same effect is achieved by rye bran-enriched wheat bread. We tested whether white wheat bread enriched with bioprocessed rye bran (BRB+WW) and sourdough wholegrain rye bread (WGR) have similar effects on glucose metabolism and plasma level of short chain fatty acids (SCFAs).

\textbf{Methods:} Twenty-one (12 women) of 23 recruited subjects completed an intervention with a four-week run-in and two four-week test periods in cross-over design. White wheat bread (WW; 3% fibre) was consumed during the run-in, and WGR and BRB+WW (10% fibre) during the test periods. A meal test providing 51/33/11 E % from carbohydrates/fat/protein was conducted at the end of each period. Fasting and postprandial plasma samples were analysed for glucose, insulin, and SCFA.

\textbf{Results:} Glucose and insulin responses and plasma concentrations of SCFAs to the meal test were similar between the WGR and BRB+WW periods. When compared to WW period, postprandial insulin concentration was lower at 120 (p=0.023) and first-phase insulin secretion improved (p=0.033) only after WGR period, whereas both rye bread periods increased concentrations of butyrate (p<0.05) or propionate (p=0.009) at 30 min postprandially.

\textbf{Conclusions:} Similar glucose, insulin, and SCFA responses to consumption of WGR were achieved with that of BRB+WW containing the same amount of fibre. Postprandially measured glucose metabolism and concentrations of SCFAs provided additional information along with fasting measurements.

9.2 BACKGROUND

Over the recent 15 years potential benefits of rye bread intake over wheat bread regarding glucose metabolism has been demonstrated. A single meal of rye bread has been repeatedly shown to reduce postprandial insulin response as compared with a single meal of white wheat bread (62,100,106), suggesting a beneficial effect on glucose metabolism. However, the data from longer-term interventions is less clear. Daily eight-week consumption of wholegrain rye bread enriched with rye bran improved first-phase insulin secretion.

measured by the frequently sampled intravenous glucose tolerance test (FSIGT) in healthy postmenopausal women (90). Similar enhancement in first-phase insulin secretion measured by the oral glucose tolerance test (OGTT) was observed in subjects with the metabolic syndrome consuming rye bread and pasta daily for 12 weeks (92). In contrast to these findings, no difference was observed in first-phase insulin secretion or insulin sensitivity in subjects with the metabolic syndrome consuming rye bread or a combination of wholegrain wheat and rye foods daily for 12 weeks, as compared to those consuming refined wheat foods (86,260).

Reduced glucose responses to a standardized meal test or OGTT have been observed in second-meal studies where a single evening meal containing high-fibre barley kernels was served to healthy subjects ten hours before the test (167,168,170,172). Furthermore, plasma butyrate or propionate concentrations were increased at breakfast when the preceding evening meal contained barley as compared to a meal with white wheat bread (167,169,170). These data suggested that butyrate and propionate that are produced by intestinal fermentation of grain fibre are associated with subsequent postprandial glucose metabolism. Regular consumption of high-fibre rye bread could also affect postprandial glucose metabolism and production of short chain fatty acids (SCFAs) after a meal test, but to date, there are no studies to support this.

Wholegrain foods are protective against type 2 diabetes and cardiovascular diseases (6), but the protective factor in wholegrain foods may be assigned to fibre-rich bran instead of wholegrains per se (261). In addition to the beneficial health effects, however, dietary fibre may cause unwanted gastrointestinal effects such as flatulence, bloating, and abdominal discomfort (262). In Finland, rye bread provides 30-50% of the total dietary fibre intake (231). Consumption of rye bread has been shown to cause gastrointestinal symptoms to some but not to all individuals (263,264). The majority of fibre in rye is cell walls polysaccharides – cellulose, arabinoxylan, and β-glucan – and fructan (a family of oligo- and polymers with degree of polymerization 3-60) (58,63). Fructan is more readily fermented than cell wall polysaccharides and the fructan content of rye might explain the appearance of gastrointestinal symptoms after rye intake (265). Furthermore, the conventional rye bread in Finland is made of wholegrain flour with sourdough fermentation, which changes nutritional quality and health effects of grain ingredients (52). Sourdough wholegrain rye bread also has a specific dense structure and sour taste, which may not appeal to consumers outside the Northern and Eastern Europe. In other parts of Europe, such as in Italy, refined grains are regarded as more tasty than wholegrains, and Italians and English perceive refined grains healthier than Finns do (266).

To increase acceptability and to reduce fermentation-derived differences between wholegrain sourdough rye bread (WGR) and native rye bran, we baked a white wheat bread (WW) that was enriched with rye bran bioprocessed with enzymes and yeast (BRB) at the same fibre level. The aim was to test whether the white wheat bread enriched with bioprocessed rye bran (BRB+WW) promotes similar effects to WGR on glucose metabolism and plasma levels of SCFAs in healthy subjects with self-reported gastrointestinal symptoms. In addition, we tested whether responses to consumption of WGR and BRB+WW differed from that of low-fibre WW.
9.3 METHODS

9.3.1 Subjects
Healthy men and women were recruited based on one or several of the following self-reported gastrointestinal symptoms after ingestion of grain products, especially rye bread: flatulence, bloating, discomfort, constipation, and diarrhea. The recruitment of subjects is described in Figure 9.1. Exclusion criteria included BMI > 35 kg/m², inflammatory bowel disease, type 1 or 2 diabetes, abnormal liver, thyroid, or renal function (hyper- or hypothyroidism and hypertension controlled with medication were allowed), fasting serum triglycerides concentration >3.5 mmol/l, fasting serum total cholesterol concentration >8 mmol/l, alcohol abuse (>16 portions/week (women)/>24 portions/week (men)), cereal or milk protein allergy, special diet (such as vegetarian or low-carbohydrate diet), and antibiotic use over the preceding two months. Transglutaminase IgA antibodies were negative (<7 U/ml) for all the subjects participating in the intervention, suggesting no celiac disease. The age, BMI, and fasting glucose of the subjects ranged from 38 to 65 years, from 19 to 30 kg/m², and from 4.9 to 6.3 mmol/l, respectively. When assessed for eligibility, subjects were informed that participation in the intervention requires daily consumption of bread over three months. Five subjects mentioned that they had reduced consumption of grain products due to gastrointestinal symptoms. Other subjects did not report changes in their habitual consumption of grain products despite the symptoms. The subjects provided written informed consent prior participating in the study. The study was approved by the Ethics Committee of the Hospital District of Northern Savo.

![Flow diagram](image)

*Figure 9.1. Flow diagram. BRB+WW, white wheat bread enriched with bioprocessed rye bran; WGR, wholegrain rye bread; WW, white wheat bread.*
9.3.2 Test breads
Refined WW were two commercial breads with 100% white wheat flour (Vaasan Oy, Finland). For baking the WGR, wholegrain rye flour was fermented with Baker’s yeast and lactic acid bacteria (Lb. brevis, Lb. plantarum) for 22 hours at 30°C. The sourdough was mixed thoroughly at the beginning of fermentation but not during the process. The sourdough was used at the 50% of substitution level in baking. The bran for the BRB+WW was fermented with enzymes and yeast and the bread was baked as previously described (267). The bread dough were left to rest for 20 min in 28 °C and 75% relative humidity, mixing twice for two and four minutes during resting. The breads were prepared in 400 g dough pieces, proofed for 50 min in 35 °C and 80% relative humidity, and baked in 225 °C for 20 min, with 15 seconds steaming in the beginning.

BRB+WW and WGR had the same total fibre content (10%) and soluble fibre content (2.1%), whereas WW had lower total fibre content (3%). Furthermore, the BRB+WW and the WGR contained 0.7% and 1.8% fructan, 0.4% and 0.7% β-glucan, and 2.0% and 1.3% soluble arabinoxylan, respectively. Using the BRB and sourdough fermentation of the wholegrain rye flour reduced the starch content of the breads (23% for BRB+WW, 32% for WGR, 44% for WW). The breads provided 10.3, 7.3, and 9.1% protein, and 3.5, 0.6, and 3.5% fat (BRB+WW, WGR, and WW respectively).

9.3.3 Study design
A four-week run-in period with low grain fibre intake preceded two consecutive four-week test periods with high grain fibre intake in randomized, cross-over manner (Figure 9.2). During the run-in period the subjects were advised to consume 6-10 slices (20-25 g/slice) of the WW daily. During the test periods, the subjects were asked to consume 6-10 slices (25-30 g/slice) of the WGR and BRB+WW daily, in randomized order. The specific amount of bread slices depended on individual energy requirement of the subjects. The test breads were provided for the subjects free of charge. The subjects were informed and followed weekly or biweekly on the practical management of the diets by a dietician. Furthermore, they were advised to maintain their body weight and follow their habitual living habits throughout the study.

For investigating gastrointestinal discomfort due to consumption of the test breads, the subjects were required to exclude other food items possibly causing symptoms during the whole three-month intervention. Dietary counseling was based on avoiding vegetables, fruits, and pulses containing readily fermentable oligo-, di- and monosaccharides and polyols as well as foods supplemented with fructo-oligosaccharide, inulin, or galacto-oligosaccharide (265). Instead, the subjects were asked to favor vegetables, fruits, and berries without or with only a low content of these fermentable carbohydrates. One to two small portions of other grain products than the test breads were allowed daily. If eating oatmeal daily over the run-in period, this was required also during the other periods. If the subjects experienced constipation over the run-in period, occasional intake of non-grain fibre supplements such as dried and soaked plums, linseeds, and sugar beet fibre were allowed.
Subjects recorded the eaten amount of the test breads and other grain products in a daily questionnaire, and filled in four-day food records over the last week of each period. The food records were analysed for nutrient intakes using Diet32 software (version 1.4.6.3, Aivo Finland Oy, Turku, Finland) which includes database of Finnish foods. Nutrient compositions of the WW, BRB+WW, and WGR were added to the database.

At screening and after each period, the subjects filled in a questionnaire inquiring their quality of life regarding gastrointestinal symptoms (gastrointestinal quality of life questionnaire, GIQLI (268)). The subjects also recorded occurrence of gastrointestinal symptoms (flatulence, bloating, rumbling of stomach, abdominal pain, and heartburn) as well as defecation frequency daily over the first and last week of each period. The occurrence of symptoms was assessed by a 5-point scale as follows: 0 no symptoms; 1 slight symptoms; 2 moderate symptoms; 3 severe symptoms; 4 very severe symptoms.

Figure 9.2. Study design. Test meal included a standardized portion of white wheat bread, margarine, cheese, cucumber, and juice. BRB+WW, white wheat bread enriched with bioprocessed rye bran; WGR, wholegrain rye bread; WW, white wheat bread.

Standardized meal test
Instead of OGTT, we used a standardized meal test for experimental purpose to study glucose metabolism because it better reflects real life situation among free living individuals. The meal test was performed after each four-week period. On the day preceding the meal test, the subjects were asked to avoid unusually large portions of food and avoid consumption of alcohol for two days before the meal test. After an overnight fast, the subjects arrived in the laboratory, their weight was measured, an intravenous catheter was inserted in the antecubital vein of the arm and a fasting blood sample was taken.

The meal included white wheat bread (80 g), milk-free margarine (20 g), cheese (20 g), cucumber (40 g), and juice concentrate (0.4 dl) diluted in 2.6 dl water. According to manufacturer’s information of each food item, the meal contained 550 kcal and 3.7 g fibre. Percentage of energy from carbohydrates, fat and protein was 51%, 33%, and 11%, respectively. Meal eating time was restricted to fifteen minutes. After starting to eat the meal, four blood samples were taken at 30, 60, 120, and 180 minutes.
9.3.4 Chemical and biochemical analyses
Chemical composition of the test breads regarding protein, fat, starch, total dietary fibre, soluble fibre and arabinoxylan, fructan, and β-glucan was determined as described by Nordlund et al (160).

Blood samples were analysed for plasma glucose and insulin concentrations as follows: glucose was analysed using Konelab 20XTi Clinical Chemistry Analyser and Enzymatic photometric (glucose hexokinase) method (Konelab System Reagents, Thermo Fisher Scientific, Vantaa, Finland), and insulin was analysed with a chemiluminescent immunoassay (Advia Centaur Immunoassay System, Siemens Medical Solution Diagnostics, Tarrytown, NY, USA). Concentration of fasting serum total cholesterol and triglycerides were analysed using commercial kits (Thermo Electron Corporation, Vantaa, Finland) and Thermo Fisher Konelab 20XTi Analyser (Thermo Electron Corporation, Vantaa, Finland). To exclude celiac disease in screening, fasting serum sample was analysed by routinely used assay for transglutaminase IgA antibodies with fluoro-enzyme immunoassay method. SCFA in 0, 30, and 180 min plasma samples were measured by gas chromatography as described by Brighenti (182) with slight modifications using 2-ethyl butyrate (FLUKA no. 03190; Sigma Aldrich, St. Louis, MO, USA) as an internal standard instead of iso-valeric acid. The internal microflora does not produce 2-ethyl butyrate, and it is consequently not present in biological samples.

9.3.5 Calculations
Area under the curve (AUC) of glucose and insulin was calculated for each meal test from the area beneath the curve above the fasting level from 0 to 120 min using GraphPad Prism 4.0 for Windows (GraphPad Software, Inc., San Diego, CA, USA). First-phase insulin secretion, which is indicative of the insulin secretion capacity with relation to plasma glucose concentration, was calculated as following: (insulin 30 min – 0 min) / (glucose 30 min – 0 min). Disposition index (DI), which represents early insulin secretion taking insulin sensitivity into account, was calculated as a product of first-phase insulin secretion and insulin sensitivity (ISIcomp; (78)): (insulin 30 min – 0 min) / (glucose 30 min – 0 min)*(10000/√((G0*I0*Gm*Im)) (G0 = fasting glucose concentration; I0 = fasting insulin concentration; Gm = mean of postprandial glucose concentrations; Im = mean of postprandial insulin concentrations). Since there was no difference in the occurrence of gastrointestinal symptoms between the first and the last week of each period, the mean of the two weeks was calculated.

9.3.6 Statistical analyses
To make data analysis of glucose and insulin responses from the meal test more similar to that from 2-hour OGTT, and because there were no statistically or clinically relevant differences in glucose, insulin, and SCFA concentrations at 180 min, results for glucose and insulin responses are reported based on 0, 30, 60, and 120 min plasma samples. Results for SCFA are reported based on 0 and 30 min plasma samples. First, glucose, insulin, and SCFA responses were compared after the WGR and BRB+WW periods. Then, the WW period was included in the analysis and the comparisons were made among the three periods. Comparisons between and among the periods regarding subjects’ characteristics, intake of nutrients, plasma glucose and SCFA concentrations, glucose AUC, first-phase insulin
secretion, and DI were conducted using General linear model (GLM) for repeated measures. For GLM, non-normally distributed variables were logarithmic-transformed. Plasma insulin concentrations, insulin AUC, gastrointestinal symptoms, frequency of defecation, energy intake, and total fibre intake were not normally distributed, and thus were compared among the periods using the Friedman’s test and between the periods using the Wilcoxon signed rank test. All analyses were conducted with SPSS 19.0 for Windows (Chicago, IL). Because of the experimental purpose of the meal test, p-values for markers of glucose and insulin metabolism are reported as non-adjusted values when the comparisons were made among the three periods. For other variables, the p-values were corrected for multiple comparisons. p-values < 0.05 were regarded statistically significant. The data is expressed as mean ± SD or mean ± SEM.

9.4 RESULTS

9.4.1 Compliance and tolerance to diet
The subjects consumed the test breads as advised based on the daily questionnaires. The intake of energy and percentage of energy from fat did not change during the intervention, whereas the percentage of energy from protein was slightly higher during the BRB+WW period as compared to the other periods (p<0.05) (Table 9.1). The percentage of energy intake from carbohydrates was lower (p<0.001) during the BRB+WW period than during the other periods due to the lower starch content of the bread. The intake of total fibre and fibre from the breads was lower during the WW period than during the other periods (p<0.001), and similar between the BRB+WW and WGR periods. Consumption of other grain products was similar over each period (1.0 ± 0.6 portions/d). Total serum cholesterol was significantly (p<0.01) higher at the end of BRB+WW than WGR period (Table 9.2).

Table 9.1. Intake of energy, nutrients, and test breads over the bread periods (n=21)

<table>
<thead>
<tr>
<th></th>
<th>WW period</th>
<th>BRB+WW period</th>
<th>WGR period</th>
<th>p-value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy, kJ/d (kcal/d)</td>
<td>8 620 ± 1 720</td>
<td>8 410 ± 2 140</td>
<td>8 210 ± 1 930</td>
<td>0.405</td>
</tr>
<tr>
<td></td>
<td>(2 060 ± 410)</td>
<td>(2 010 ± 510)</td>
<td>(1 960 ± 460)</td>
<td></td>
</tr>
<tr>
<td>Carbohydrates, E%</td>
<td>43 ± 6</td>
<td>38 ± 7***</td>
<td>42 ± 7###</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Protein, E%</td>
<td>20 ± 3</td>
<td>22 ± 3*</td>
<td>20 ± 3#</td>
<td>0.003</td>
</tr>
<tr>
<td>Fat, E%</td>
<td>33 ± 5</td>
<td>35 ± 6</td>
<td>32 ± 6</td>
<td>0.094</td>
</tr>
<tr>
<td>Total fibre, g/d</td>
<td>21 ± 7</td>
<td>34 ± 10***</td>
<td>33 ± 10***</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Bread, g/d</td>
<td>169 ± 24</td>
<td>195 ± 53</td>
<td>205 ± 50***</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Fibre from bread, g/d</td>
<td>5 ± 1</td>
<td>20 ± 5***</td>
<td>21 ± 5***</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

† Among the bread periods (GLM for repeated measures adjusted for multiple comparisons; for energy and total fibre Friedman’s test followed by Wilcoxon signed rank test adjusted for multiple comparisons). Different from WW period: *p<0.05, ***p<0.001. Different from BRB+WW period: #p<0.05, ###p<0.001. BRB+WW, white wheat bread enriched with bioprocessed rye bran; E%, percentage of total energy intake; WGR, wholegrain rye bread; WW, white wheat bread. Data are presented as mean ± SD.
Table 9.2. Characteristics of the subjects at end of each bread period (n=21)

<table>
<thead>
<tr>
<th></th>
<th>WW period</th>
<th>BRB+WW period</th>
<th>WGR period</th>
<th>p-value(^1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (kg)</td>
<td>72 ± 14</td>
<td>72 ± 13</td>
<td>72 ± 14</td>
<td>0.133</td>
</tr>
<tr>
<td>Fasting glucose (mmol/l)</td>
<td>5.1 ± 0.4</td>
<td>5.0 ± 0.5</td>
<td>5.0 ± 0.5</td>
<td>0.449</td>
</tr>
<tr>
<td>Cholesterol (mmol/l)</td>
<td>5.2 ± 0.9</td>
<td>5.4 ± 0.9</td>
<td>5.0 ± 0.7##</td>
<td>0.006</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>0.9 ± 0.4</td>
<td>0.9 ± 0.4</td>
<td>1.0 ± 0.6</td>
<td>0.499</td>
</tr>
</tbody>
</table>

\(^1\) Among the bread periods (GLM for repeated measures adjusted for multiple comparisons). Different from BRB+WW period: ##p<0.01. BRB+WW, white wheat bread enriched with bioprocessed rye bran; WGR, wholegrain rye bread; WW, white wheat bread. Data are presented as mean ± SD.

At screening, the GIQLI score was 119 ± 12, while at the end of the WGR and BRB+WW periods the score was 125 ± 10 and 126 ± 9, respectively. The subjects reported significantly less gastrointestinal symptoms over the 4-week run-in (127 ± 9) and test periods than at the screening when they followed their habitual diet (p<0.01). They experienced more often slight or moderate flatulence over the BRB+WW and WGR periods than over the WW period (p<0.05). Altogether 24% (n=5) and 29% (n=6) of the subjects experienced moderate or severe flatulence over BRB+WW and WGR periods, respectively, while only one subject experienced moderate or severe flatulence during the WW period. There were no differences in experiencing bloating, rumbling of stomach, abdominal pain, or heartburn among the periods, and over 90% of the subjects reported none or slight symptoms. Frequency of defecation was not affected by the period, being on average 1.4 times per day.

9.4.2 Glucose and insulin

Fasting and postprandial glucose and insulin responses to the meal test were similar between the WGR and BRB+WW periods (Figure 9.3), as well as glucose and insulin AUCs and first-phase insulin secretion (data not shown), and disposition index (DI) (3519 ± 4947 and 3614 ± 2883 for BRB+WW and WGR periods, respectively). In further analysis, response of plasma insulin to the meal test was lower after the WGR period than after the WW period at 120 min (p=0.023) (Figure 9.3 B). First-phase insulin secretion to the meal test tended to differ among the periods (p=0.083). DI differed among the periods (p=0.042), showing a difference between the WW and WGR periods (p=0.033; 2500 ± 1336 and 3614 ± 2883, respectively).
9.4.3 Plasma SCFA
Fasting concentration of total SCFAs (sum of acetate, propionate, and butyrate) was the same among the periods (mean ± SD of all periods: 89 ± 26 μmol/L). Also the fasting acetate, propionate, and butyrate concentrations did not differ among the periods. Acetate concentration accounted for 94% of the total concentration of SCFAs. From fasting to 30 min postprandially, the total SCFA, acetate, propionate, and butyrate concentrations were similar between the WGR and BRB+WW periods (p>0.345 for all; time x period interaction). When comparisons were made among all the three periods, propionate concentration tended to differ (p=0.058 for time x period interaction) and butyrate concentration differed (p=0.011 for time x period interaction) from fasting to 30 min postprandially (Figure 9.4 A, B). Propionate concentration was higher after the WGR period than after the WW period at 30 min (p<0.01). At 30 min, butyrate concentration was higher after the WGR (p<0.01) and BRB+WW periods (p<0.05) than after the WW period.

Figure 9.3. Fasting and postprandial A) glucose and B) insulin responses to the meal test following the four-week periods with consumption of the different test breads (mean ± SEM, n=21). *p<0.05 between the WGR and WW periods (Wilcoxon signed rank test). BRB+WW, white wheat bread enriched with bioprocessed rye bran; WGR, wholegrain rye bread; WW, white wheat bread.
9.5 DISCUSSION

In the present study, we compared the effects of four-week consumption of WGR and BRB+WW on postprandial glucose and insulin responses and plasma concentrations of SCFA after a standardized meal test. We observed that enrichment of white wheat bread with bioprocessed rye bran produced similar effects to WGR on glucose metabolism and plasma concentrations of SCFAs.

Although BRB+WW included white wheat flour in addition to rye bran, it had the same fibre content as WGR which was baked from wholemeal rye flour. The similar fibre content might explain the similar effects. WGR provided 2.5-fold more fructan (3.7 vs. 1.4 g/d) than
BRB+WW bread, but inulin-type fructans do not seem to affect fasting glucose concentration in humans (269), and there is no evidence that approximately 2 g more fructan per day would affect insulin metabolism. Reduction in the two-hour postprandial insulin concentration and increment in first-phase insulin secretion, when subjects' insulin sensitivity was taken into account, were observed only following consumption of WGR as compared to that of WW. The results suggest that first-phase insulin secretion was improved so that less insulin was needed in the later postprandial phase to control glucose concentration. Thus, it seems that WGR might have additional effects that are independent of the total fibre content of bread. In support of this postulation, a single rye bread meal has been shown to reduce postprandial insulin response in healthy subjects as compared to that of white wheat bread, and this effect is not related to the fibre content of the rye bread (90,100,106). Sourdough fermentation does not seem to explain the effect either since also endosperm and wholegrain rye breads baked without sourdough fermentation process reduced the acute postprandial insulin response (100). Furthermore, BRB+WW bread did not have similar beneficial effect on the acute insulin response in our recent postprandial study (267). Improved first-phase insulin secretion and subsequent reduced hyperinsulinemia in later postprandial phase may prevent disturbances in glucose metabolism (270).

To our knowledge we are the first who have used a meal test to investigate glucose metabolism after a longer-term regular intake of rye bread or rye bran enriched wheat bread. Methodological differences and differences in study populations may explain the inconsistent results from previous interventions on wholegrain or fibre-rich rye bread. A recent study reported that 12-week consumption of sourdough-fermented rye and wholemeal wheat breads and other wholegrain products had no effect on glucose and insulin metabolism measured with the FSIGT (260). The authors postulated that effects of wholegrains on insulin sensitivity may be mediated by gastrointestinal hormones secreted postprandially after a meal, a condition which is not met in the FSIGT. This speculation is supported by several intervention studies showing no effect on fasting glucose and insulin concentration by consumption of rye bread as compared to refined wheat bread (86,90,94,95). Unlike FSIGT, OGTT takes into account the postprandial events. However, no difference was observed in glucose and insulin responses (measured as AUCs) or 2-hour glucose and insulin concentrations in OGTT performed after 12 weeks of diet containing rye bread or rye bread and pasta as compared with diet containing refined wheat bread or oat and wheat bread and potatoes, respectively (86,92). Instead, a study with subjects with insulin resistance showed decreased 2-hour glucose and insulin concentrations in OGTT after 6-week consumption of sourdough wholegrain wheat bread as compared to that of white wheat bread (91). In OGTT only glucose is ingested and no digestion is required, whereas the standardized meal test, as used in the present study, mimics the true physiological event of ingestion of a mixture of foods that are digested to nutrients and absorbed and may differently affect gastrointestinal hormones.

The above interventions were conducted with subjects with features of the metabolic syndrome and elevated fasting glucose or insulin concentration, whereas in the present study we had subjects with, on average, normal fasting glucose concentration. Laaksonen et al. (92) have reported that consumption of rye bread-based diet improves first-phase insulin
secretion in OGTT as compared to the wheat-based control diet. Also an improvement in first-phase insulin secretion in OGTT was observed in subjects with normal glucose tolerance after consumption of sourdough wholegrain wheat bread vs. white wheat bread (91). In agreement with these findings, we observed improved first-phase insulin secretion after consuming WGR as compared to that of WW when possible variation in insulin sensitivity between the subjects was taken into account.

The increase in fasting cholesterol due to consumption of the bread containing bioprocessed rye bran might be explained by the slightly, although not statistically significant, higher intake of fat over the BRB+WW period. However, this requires further investigation because similar effect of (non-processed) rye bran-enriched bread on serum cholesterol was observed previously in postmenopausal women without change in fat intake (271).

We observed no differences in concentrations of fasting plasma total SCFA or individual SCFA between the rye-containing and WW diets, supporting a previous finding (177) that consuming high-fibre rye bread does not seem to affect fasting plasma level of SCFA in humans. However, rapid increases in the concentration of plasma butyrate after the meal were observed following consumption of the both rye-containing breads, while consumption of WGR also increased the concentration of propionate as compared to WW. Increased butyrate concentrations due to consumption of rye as compared to wheat have been observed before, when measured from human faeces (95,176), in the portal vein of pigs (178,179), and in the peripheral blood of pigs (177). We propose that it is possible to observe the effects of intestinal fermentation on plasma concentrations of SCFA in humans when measured in the postprandial state.

In the present study for WGR period, the improvement in insulin responses postprandially appeared to occur simultaneously with the increased plasma concentrations of propionate and butyrate. In the studies of Nilsson et al (167,169) and Priebe et al (170), increase in plasma propionate and/or butyrate concentrations occurred simultaneously with reduction in postprandial glucose response to a standardized meal the following morning after a barley evening meal. Regarding rye, feeding rye bread to pigs increased absorption of butyrate from the intestine and lowered insulin response suggesting improved insulin sensitivity either at the liver level or in muscles (179). Increase in insulin sensitivity has also been associated with increase in butyrate producing intestinal bacteria in subjects with metabolic syndrome (131). However, our study does not unambiguously support the previously observed associations between butyrate and glucose metabolism, because also consumption of the BRB+WW increased plasma concentration of butyrate at 30 min without significant improvement in the postprandial insulin responses as compared to WW. On the other hand, we cannot rule out that increased concentration of plasma propionate after consumption of WGR could improve glucose metabolism.

In our study, self-reported gastrointestinal symptoms seemed not to limit the consumption of the rye-containing breads. Surprisingly, subjects’ quality of life regarding gastrointestinal symptoms increased during the controlled consumption of all the test breads. This might be due to avoidance of readily fermentable carbohydrates in certain vegetables, fruits, pulses,
and fibre-supplemented food products (265). Intake of the rye-containing breads where most of the fermentable carbohydrates existed as cell wall polysaccharides seemed to be more tolerable when the total load of the readily fermentable carbohydrates was reduced. The rye-containing breads caused flatulence which, however, did not decrease subjects’ quality of life.

In conclusion, similar effects of consumption of WGR on glucose metabolism and plasma concentration of SCFA were achieved with white wheat bread that was enriched with bioprocessed rye bran. Furthermore, the study emphasizes the importance of postprandial measurements of glucose metabolism and plasma concentrations of SCFA in addition to fasting measurements.
10 General Discussion

10.1 RESULTS

10.1.1 Acute effects in postprandial studies
Postprandial settings in Studies I and IV investigated short-term effects of wholemeal and fibre-rich breads on glucose metabolism, and on absorption of FA and its metabolites from the small and large intestine. As compared to white wheat bread, postprandial glucose and insulin responses were reduced by ingestion of wholemeal wheat bread that was baked with sourdough fermentation. There were no differences in the postprandial responses between the white wheat and conventional wholemeal wheat breads. The results of this study are in accordance with those of previous studies, in which sourdough fermentation of white wheat bread or wholemeal wheat bread reduced glucose and/or insulin responses as compared to corresponding breads baked with straight dough (104,114-116). No difference has been observed in postprandial responses between the conventional wholemeal wheat and white wheat breads (103,104,116). Study I also aimed to investigate factors underlying the postprandial responses. The content of fibre of the wholemeal wheat breads did not explain the reduced responses for the sourdough bread. In vitro digestion of protein and content of soluble protein as well as molecular weight of soluble arabinoxylan in the breads were investigated but these did not provide any explanation for the reduced postprandial responses for the sourdough-fermented wholemeal wheat bread.

Enrichment of white wheat bread with native or bioprocessed rye bran did not improve postprandial glucose and insulin responses as compared to white wheat bread. This result is similar to what has been reported earlier for high-fibre white wheat bread enriched with native rye bran (100). However, Study IV showed that wholegrain rye bread reduced postprandial insulin response, confirming the observations from previous studies with healthy subjects (62,100,105-107). Rye bread does not need to be made of wholegrain flour because a similar reduction in the postprandial insulin response has been observed with endosperm rye bread (61,62,100,107). Furthermore, the content of fibre is not the determinant of the low postprandial insulin response because there is a large variation in the fibre content of the breads (from 5.5% to 14.5%),which reduces the postprandial insulin response (62). Breads enriched with rye bran and containing 10% and 11.6% of fibre did not reduce the insulin response in Study IV.

The reduced insulin response after intake of rye bread could be explained by differences in the metabolic processes occurring postprandially. The firm structure of rye bread might retard digestion and absorption of starch, which in turn reduces insulin response. However, the concurrently observed lack of difference in glucose response needs explanation. For instance, the retarded uptake of glucose by tissues counterbalancing reduced absorption may thereby maintain blood glucose concentration. This hypothesis is based on recent studies with $^{13}$C-labeled starch and dual isotope technique, which show that postprandial plasma glucose concentration does not necessarily reflect the rate of digestion and absorption of a starchy food (272). Blood glucose concentration after a
glucose-containing meal is determined by the rate of absorption of glucose from the intestine, endogenous glucose production by the liver, and uptake of glucose by the tissues. A postprandial study was conducted to clarify these phenomena after ingestion of bran-enriched wheat bread and bran-enriched wheat pasta (273). Both the wheat bread and pasta produced similar postprandial glucose responses, but the insulin response was reduced only after pasta. Intake of pasta reduced the rate of appearance of glucose into the circulation as compared to the intake of bread. However, blood glucose concentration was not reduced after pasta intake, and this was because of a slower glucose clearance rate by the tissues.

As the conclusion of their elaborate study (273) the authors suggest that there are three different kinds of starchy products: those with rapidly digestible starch, leading to high postprandial glucose and insulin responses; those with slowly digestible starch, leading to high postprandial glucose response but reduced insulin response; and those with very slowly digestible starch, leading to low postprandial glucose and insulin responses. Thus, rye bread would be classified as a slowly digestible starch product.

Bioprocessing of rye bran in Study IV increased the absorption of free FA from the small intestine, but increased absorption of FA did not affect postprandial responses. A similar effect of bioprocessing on the absorption of free FA has been observed previously with wheat bran (153). The test bread portions provided at breakfast and lunch contained 20 g of grain fibre and were planned to be equivalent to the intake of grain fibre and grain phenolic acids during a whole day in a normal diet. Bioprocessing of rye bran in Studies IV and V also aimed to loosen and break down the fibre structure in the bran to induce more active and complete fermentation and fibre degradation in the large intestine. However, no increment was observed in the urinary excretion of the metabolites of FA after ingestion of white wheat bread enriched with bioprocessed rye bran as compared with the other rye-containing test breads. Also, ingestion of the conventional wholegrain rye bread providing 20 g of fibre did not change the excretion of these metabolites as compared with that of white wheat bread (providing only 5 g fibre during the test day) when the subjects followed their habitual background diet. This suggests that neither high nor low intake of grain fibre during one day can modify the circulating pool of phenolic acid metabolites when phenolic-rich food items are regularly consumed in the habitual diet.

10.1.2 Long-term effects in intervention studies
Although postprandial studies provide information about the short-term effects of grain products on glucose metabolism, it is not well known whether these effects, when repeated daily, affect glucose metabolism in the longer-term. That is why we investigated glucose metabolism over twelve and four weeks (in Studies II and V, respectively) of daily consumption of the wholegrain foods and fibre-rich breads. Furthermore, the composition of intestinal microbiota and the plasma concentration of SCFAs were studied as possible large-intestinal effects in relation to glucose metabolism.

Regular twelve-week consumption of sourdough-fermented wholemeal wheat bread, high-fibre rye breads, and other wholegrain foods by subjects with metabolic syndrome did not improve insulin sensitivity measured intravenously. The result is in accordance with
intervention studies with wholegrain and fibre-rich wheat or rye products in which insulin sensitivity was also measured intravenously with the FSIGT (90) or euglycemic hyperinsulinemic clamp (89), or evaluated at the fasting state (83-87). Only one study with hyperinsulinemic subjects has shown that consumption of wholegrain wheat foods as compared with that of refined wheat foods improves insulin sensitivity when measured intravenously by the euglycemic hyperinsulinemic clamp (81). Based on Study II and other studies on wholegrain foods, it seems that consumption of wholegrain and fibre-rich grain foods has no effect on insulin sensitivity over a period of 3 to 16 weeks in healthy subjects or in subjects with metabolic syndrome. However, this does not rule out that wholegrain foods and foods rich in grain-fibre could affect factors of glucose metabolism in the longer term. In fact, the follow-up time in studies showing decreased appearance of type 2 diabetes associated with consumption of wholegrain foods in the prospective cohort studies has ranged from 6 to 18 years (see Table 2.1, p. 7 for the references). Thus, consumption of wholegrain foods and foods rich in grain fibre can still be recommended because there are no intervention studies showing detrimental effects of these food items on glucose metabolism and because prospective cohort studies advocate this consumption for prevention of type 2 diabetes.

No effect on first-phase insulin secretion was observed in Study II. This result is in conflict with the previous finding that first-phase insulin secretion, when also measured with FSIGT, is improved after consumption of high-fibre rye breads in healthy subjects with mainly normal glucose metabolism and without cholesterol-lowering medication (90). When evaluated using the OGTT, consumption of high-fibre rye breads improved first-phase insulin secretion in subjects with the metabolic syndrome and without cholesterol-lowering medication (92), but not when the medication was allowed (86). First-phase insulin secretion measured in OGTT was improved also in healthy subjects with normal glucose tolerance after consumption of sourdough wholemeal wheat bread (91). Study V showed improved first-phase insulin secretion in healthy subjects after consumption of rye bread as compared with that of white wheat bread, when using the standardized meal test instead of OGTT to measure glucose metabolism. The results of Studies II and V together with those of previous studies suggest that consumption of sourdough wholemeal wheat or high-fibre rye breads might produce detectable improvement in first-phase insulin secretion only in healthy individuals or in individuals with disturbances in glucose metabolism not using cholesterol lowering medication. It should be noted, however, that the standardized meal test is not a generally accepted clinical method to measure glucose metabolism, and therefore further studies are needed to confirm the observations with the meal test as compared with OGTT.

A twelve-week consumption of the wholegrain diet containing the wholegrain and fibre-rich rye breads did not affect intestinal microbiota composition as compared to that of refined grain diet based on white wheat breads in subjects with the metabolic syndrome. The subjects in Study III had habitually consumed high-fibre rye bread prior to entering the study. A diet followed over several years probably affects the composition of the intestinal microbiota (216-218). An individual’s initial microbiota composition seems to affect responses to changes in diet (143,217). Thus, it is not known whether there would have been differences in the composition of the intestinal microbiota if the subjects had
habitually consumed a refined grain-based diet from which they switched to a wholegrain based diet.

It is also questionable whether the changes in the composition of intestinal microbiota reflect changes in microbiota metabolism. Regarding the physiological events within the gut, the functions of the intestinal microbiota may be more important than the composition. ‘Functional redundancy’ is referred to when discussing several different phytotypes which perform the same metabolic tasks and thus ensure stability in the gut when conditions change (122). Intestinal microbiota functions are beyond the scope of this doctoral thesis.

Concentration of butyrate in plasma increased postprandially after a four-week consumption of rye-containing breads as compared with that of white wheat bread. Our data from study V does not allow us to conclude whether butyrate or propionate can improve glucose metabolism in a cause-and-effect manner; neither does the data from the second-meal studies investigating the association of SCFAs with glucose metabolism (167,169,170). Instead, an increase in postprandial plasma concentrations of butyrate and propionate occurred simultaneously with an improvement in first-phase insulin secretion and a reduced need for insulin in the later postprandial phase in the low-fibre meal test only after consumption of wholegrain rye bread. There are hypotheses based on in vitro and animal studies that circulating SCFA could improve glucose metabolism via anti-inflammatory effects (165,166). In further studies, postprandial plasma concentrations of SCFAs should be measured in addition to fasting state ones when investigating large intestinal fermentation of grain fibre.

10.2 METHODOLOGICAL CONSIDERATIONS

10.2.1 Study populations
The study populations included in this doctoral thesis consisted of subjects with different characteristics. In all, the subjects were healthy adults aged approximately 60 years, the health status confirmed by laboratory analyses and clinical examination at screening. However, the state of the subjects’ glucose metabolism varied. In Studies I, II, and III, the subjects had IFG and/or IGT and metabolic syndrome, while in Studies IV and V, the subjects were not recruited based on the status of glucose metabolism but based on self-reported gastrointestinal symptoms. Thus, the latter studies included subjects with normal glucose tolerance or IFG (range of the fasting glucose concentration from 4.9 to 7.1 mmol/l). The OGTT was only performed at screening in Study II, so there is no baseline data from the other studies whether the subjects had IGT. Furthermore, only Study II provides information about subjects’ fasting insulin concentration at baseline.

All the subjects were women and men from Kuopio, except in Study II in which 44% of the subjects were from Naples, Italy. The published intervention and postprandial studies included both men and women, mainly from Finland, Sweden, USA, and UK (see Tables 2.5 and 2.6; p. 15-19 and 22-24). Power calculation to evaluate adequate sample size was done only in Study II, and the number of subjects in this study was adequate for detecting a clinically relevant effect on insulin sensitivity. In the postprandial studies, the number of
subjects was eleven and fifteen in Study I and IV, respectively. Previously, postprandial differences in insulin response between ingestion of rye and white wheat breads have been observed with 10-20 healthy subjects (62,100,105,107), suggesting that the sample size in Studies I and IV was adequate. In Study III, it is difficult to evaluate an adequate sample size because of the large inter-individual variation in intestinal microbiota composition. However, the number of subjects was almost the same as in the only other parallel study with grain-based diets and with 69 overweight subjects (147). The other grain-based studies investigating the composition of the intestinal microbiota followed a cross-over design and included 14-39 subjects (96, 141-146). In Study V, 33% of the subjects had IFG (according to ADA criteria (70) while the rest had normal glucose tolerance. The study followed a cross-over design, the subjects acting as their own controls, which reduces the number of subjects needed as compared to a parallel study design. However, because of the large inter-individual variation in the concentration of fasting glucose, the statistical power of the study might have been insufficient to observe all clinically relevant differences in the markers of glucose metabolism. Furthermore, probably due to large inter-individual variation in postprandial glucose and insulin responses to the white wheat bread enriched with bioprocessed rye bran, the responses did not statistically differ from those to white wheat bread.

A notable aspect is that in Study II, 29% of the subjects from Kuopio had cholesterol-lowering medication. Statins are suspected to affect glucose metabolism, slightly increasing the risk of type 2 diabetes (274). Although the number of subjects using cholesterol lowering medication was the same in the wholegrain and control groups (n=10 for both groups), the use of statins among the Kuopio subjects might have been a confounding factor when analysing insulin sensitivity.

10.2.2 Test products
The aim of this doctoral thesis was to investigate the effects of wholegrain foods and grain fibre on glucose metabolism. The emphasis was on wheat and rye breads while Studies II and III included also a smaller proportion of other wholegrain foods. In Studies III, IV, and V (rye-containing high-fibre breads) more focus was on rye than wheat, while in Study II rye products were consumed in combination with wheat products. Only Study I investigated merely wholemeal wheat breads. Furthermore, the effects regarding intestinal microbiota composition, phenolic acid absorption, and SCFA were studied only with rye products. However, in all of the studies, low-fibre white wheat bread containing 3-4% fibre was used as a control product.

Results from the earlier intervention studies also apply both to grain fibre and wholegrain foods, though only 4/14 studies (83,89,95,96) have reported the content of wholegrains in their test diets (see Table 2.5; p. 15-19). Thus, the effects of wholegrains per se might be mistaken for those of grain fibre. One of the studies (Study I) investigated wholemeal breads fulfilling the criterion for wholegrain ingredients, i.e. containing bran, germ, and endosperm in the same relative proportions as in the intact kernel and allowing the removal of less than 10% of the bran (11). In addition to being made of wholegrain, the wholemeal wheat breads were high in fibre (6.5%). In Studies II and III wholegrain breads were used but in combination with other wholegrain products and high-fibre endosperm..
rye bread (7% fibre) which of course is not a wholegrain food. Studies IV and V investigated the effects of grain fibre by using high-fibre white wheat breads which contained rye bran and approximately 10% grain fibre, and wholegrain rye bread. In the European Union, bread can be called high-fibre when it contains at least 6% of fibre (275). Study V suggests that grain fibre might indeed mediate the longer-term effects of wholegrains, since daily intake of rye bran in wheat bread matrix and wholegrain rye bread produced the same effects on fasting glucose and glucose and insulin responses to a standardized meal. However, the improved insulin response, as compared with that of white wheat bread, was observed only after consumption of wholegrain rye bread, suggesting that also rye bread matrix is important for the effect. In the future, studies should focus not only on the effects of grain fibre and wholegrains but also on that of food structure.

To investigate whether baking technology influences glucose and insulin responses in postprandial studies, breads were made using the traditional sourdough fermentation method, the straight dough method (i.e. usual yeast-leavening), and bioprocessing with enzymes and yeast. Sourdough-fermented wholemeal wheat bread, white wheat bread enriched with bioprocessed rye bran, and conventional sourdough wholegrain rye bread were used also in the interventions. However, the consumed amount of the sourdough wholemeal wheat bread was small (approximately one slice per day) in the Kuopio study centre, while the subjects in Naples consumed approximately five slices per day of wholemeal wheat bread that was also baked with sourdough fermentation. Thus, Study II provided information about the effects of sourdough wholemeal wheat breads on glucose metabolism, in combination with sourdough-fermented rye breads and other wholegrain products. Study V provided information about the effects of the daily consumption of white wheat bread enriched with bioprocessed rye bran and sourdough-fermented wholegrain rye bread on glucose metabolism. Other interventions have not used (or do not report using) wholegrain or high-fibre breads made by sourdough fermentation, except for one Canadian study (91) and one Finnish study (86), in which sourdough-fermented wholegrain wheat bread was included in the test diet. It could be assumed that rye breads in the Finnish intervention studies (86,90,92,94) were sourdough fermented, but the bread baking method is not described.

In addition to ingredients, food structure is known to influence starch digestibility (276). Thus, it is important to better specify the types of breads used in intervention studies.

10.2.3 Measurement of glucose metabolism
Short-term glucose metabolism was measured postprandially in Studies I and IV and longer-term glucose metabolism by the FSIGT and the standardized meal test in Studies II and V, respectively. Postprandially, glucose metabolism was evaluated from overall glucose and insulin responses among the ingested test breads over four hours. Furthermore, glucose and insulin concentrations at individual time points, glucose and insulin AUCs, and maximum increases in glucose and insulin (only in Study I), and GP (only in Study IV) were determined. These methods of investigating postprandial responses and the form of the glucose and insulin curves are commonly used in postprandial studies.
The FSIGT is a generally accepted method to measure insulin sensitivity and first-phase insulin secretion (78). The indices calculated from the FSIGT were SI, QUICKI, DI, and dAIRg (2-10 min). QUICKI and SI evaluated insulin sensitivity in fasting state and during the 3-hour intravenous test, respectively. DI and dAIRg evaluated first-phase insulin secretion.

In Study V, a standardized meal test was performed instead of the OGTT to measure postprandial glucose metabolism under true physiological conditions of ingestion of a meal containing several nutrients in free-living individuals. First-phase insulin secretion (i.e. IGI) and DI were calculated in a manner similar to that in the OGTT. However, the standardized meal test is not a clinically accepted method to measure glucose metabolism, although it is commonly used to study glucose metabolism, for example in second-meal studies (167,168,171).

10.2.4 Statistical issues
The data analyses were performed with SPSS for Windows software (versions 11.5, 14.0 and 19.0), except in Study III where R (version 2.15.1) was used by a biostatistician. P-values < 0.05 were used as a criterion for statistical significance. The most used statistical test in this doctoral thesis was GLM for repeated measures — in Studies I, II, IV, and V. GLM allows us to evaluate overall effects of a treatment over time providing p-values for treatment × effect interactions. When there is an overall difference among treatments, GLM with post-hoc analyses enables us to compare effects at individual time points between treatments. GLM was not suitable for the analyses where the variables were not normally distributed. In this case, nonparametric Friedman’s test and Wilcoxon’s test were used because they do not require normal distribution of variables under testing. The nonparametric tests are more conservative than the GLM, and thus may fail to find small differences between the tested groups.

In Study IV, the linear mixed model was used along with GLM. Basically both studies provide similar results. The linear mixed model was used because by using histograms it enables less-laborious testing of the normality of variables. Furthermore, fasting values were used as covariates in the linear mixed model, when the fasting values differed among the treatments.

Multiple comparisons were corrected with Bonferroni or Benjamini-Hochberg FDR. Bonferroni correction is a conservative method and may ‘overcorrect’ p-values, thus providing false negative results. FDR correction is not that conservative and was used in Study III to convert initial q-values to the reported p-values. Correction for multiple comparisons is a prerequisite to minimize false positive results in studies with multiple variables such as in Study III where the effect of the dietary intervention was evaluated on several intestinal phylotypes making several comparisons simultaneously. However, in Study V, multiple comparisons were not corrected when analysing glucose metabolism because of the small number of comparisons and the experimental procedure of the meal test.
11 Conclusions

Acute postprandial glucose or insulin responses were improved by intake of sourdough wholemeal wheat bread and high-fibre wholegrain rye bread as compared to that of white wheat bread, confirming similar findings from previous studies. However, bioprocessed rye bran in wheat bread did not significantly affect these responses. Despite the beneficial short-term effects, insulin sensitivity, a marker of long-term glucose metabolism, was not improved by regular consumption of sourdough wholemeal wheat bread, high-fibre rye breads, and other wholegrain foods in subjects with the metabolic syndrome. Similar effects after consumption of sourdough wholegrain rye bread on glucose metabolism and plasma concentration of SCFAs were achieved by white wheat bread enriched with bioprocessed rye bran, suggesting that grain fibre might partly mediate the effects of wholegrains.

Increased small-intestinal absorption of FA due to bioprocessing of rye bran did not significantly affect glucose metabolism. Furthermore, it is unlikely that phenolic acid metabolites produced postprandially from grain fibre play a significant role in maintaining the circulating pool of these metabolites when the long-term diet is high in food items containing phenolic acids. This study showed for the first time that the composition of the intestinal microbiota was the same when consuming wholegrain and high-fibre rye breads or white wheat breads, and it is unlikely that change in the microbiota composition is necessary to modify glucose metabolism. Although prolonged consumption of wholegrain rye bread and bioprocessed rye bran increased plasma concentrations of butyrate and/or propionate after a single low-fibre meal, further studies are needed to investigate whether this finding plays a significant role in glucose metabolism. In future nutritional intervention studies, postprandial responses to a standardized meal test should be utilized in addition to fasting measurements of glucose metabolism and plasma concentrations of SCFA.

The present study suggests that effects of wholegrains and grain fibre in bread on glucose metabolism are mediated via short-term postprandial glucose and insulin responses, rather than via the studied effects in the large intestine.
12 References


35. European Food Safety Authority (EFSA). Scientific opinion on the substantiation of health claims related to whole grain (ID 831, 832, 833, 1126, 1268, 1269, 1270, 1271, 1431) pursuant to article 13(1) of Regulation (EC) No 1924/2006. EFSA Journal 2010;8:1766.


160. Nordlund E, Aura AM, Mattila I, Kosso T, Rouau X, Poutanen K. Formation of phenolic microbial metabolites and short-chain fatty acids from rye, wheat, and oat bran


192. AOAC Official Methods of Analysis (1990) No. 985.29 "Total dietary fiber".


263. Holma R, Hongisto SM, Saxelin M, Korpela R. Constipation is relieved more by rye bread than wheat bread or laxatives without increased adverse gastrointestinal effects. J Nutr 2010;140:534-541.


Appendices

APPENDIX 1. Study II.

*Appendix table.* Effects of wholegrain foods on insulin sensitivity in intervention studies

<table>
<thead>
<tr>
<th>Reference</th>
<th>Characteristics of participants</th>
<th>Study design</th>
<th>Wholegrain products (intake/d)</th>
<th>Control products (intake/d)</th>
<th>Methods for the evaluation of insulin sensitivity</th>
<th>Insulin sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pereira et al. 2002</td>
<td>6 F, 5 M hyperinsulinemic subjects (25-56 y) BMI 30 ± 1 kg/m²</td>
<td>Randomized crossover 2 x 6 wk separated by a 6-9 wk washout</td>
<td>Breakfast cereal, bread, pasta, muffin, cookies, snacks (80% of consumed grains were wheat, remainder oats, rice, corn, barley, rye). Grains were ground to flour. (6-10 servings)</td>
<td>Refined wheat, rice, and corn (no bran, germ, little fibre)</td>
<td>Euglycemic hyperinsulinemic clamp</td>
<td>↑</td>
</tr>
<tr>
<td>Juntunen et al. 2003</td>
<td>20 healthy females (3 with IGT) (59 ± 6 yrs) BMI 28 ± 3 kg/m²</td>
<td>Randomized crossover 2 x 8 wk separated by a 8 wk washout</td>
<td>High fibre rye bread (includes added rye bran) Minimum of 4-5 portions (24.1-28.1 g), (&gt; 96-140 g)</td>
<td>White wheat bread Amount (&gt; 83 -125 g)</td>
<td>FSIGT</td>
<td>←</td>
</tr>
<tr>
<td>McIntosh et al. 2003</td>
<td>28 M overweight subjects (40-65 y) BMI 30 ± 1 kg/m²</td>
<td>Randomized crossover 3 x 4 wk No washout</td>
<td>Rye WG diet: 135 g wholemeal bread, 22 g crisp bread, 50 g breakfast cereal</td>
<td>Low fibre foods: white bread (135 g), refined wheat crisp bread (42 g), rice cereal (50 g)</td>
<td>Fasting insulin and glucose concentrations</td>
<td>←</td>
</tr>
<tr>
<td>Andersson et al. 2007</td>
<td>22 F and 8 M subjects with one or more abnormalities of MetS (59 ± 5 yrs) BMI 28 ± 2 kg/m²</td>
<td>Randomized crossover 2 x 6 wk separated by a 6 – 8 wk washout period</td>
<td>3 portions of bread (45 g), 2 portions of crisp bread (12 g), 1 portion of muesli (35 g), 1 portion of pasta (70 g) Planned intake of WG ingredients = 112 g of which &gt; 90 % was consumed by the subjects Grains (wheat, rye, oat) contained &gt; 50 % WG per dry substance, mainly in milled form</td>
<td>Refined wheat, rye, and corn</td>
<td>Euglycemic hyperinsulinemic clamp</td>
<td>←</td>
</tr>
</tbody>
</table>

(Table continues on the next page)
### Appendix table (continues)

<table>
<thead>
<tr>
<th>Reference</th>
<th>Characteristics of participants</th>
<th>Study design</th>
<th>Wholegrain products (intake/d)</th>
<th>Control products (intake/d)</th>
<th>Methods for the evaluation of insulin sensitivity</th>
<th>Insulin sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Katcher et al. 2008</td>
<td>23 F and 24 M subjects with MetS: WG group (45 ± 8 y), BMI 36 ± 4 kg/m²&lt;br&gt;RG group (47 ± 10 y) BMI 36 ± 5 kg/m²</td>
<td>Randomized parallel, 12 wk&lt;br&gt;Hypocaloric diet</td>
<td>Bread and rolls, ready-to-eat cereal, brown rice, oatmeal, pasta, salty snacks and snack bars (WG was listed as the first ingredient on the food label) About 5 servings 1 serving was 1 slice of bread, 28 g of ready-to-eat cereal, or 120 ml of cooked cereal/rice/pasta</td>
<td>Refined grains (&lt; 0.2 servings of WG foods/d)</td>
<td>OGTT&lt;br&gt;Fasting and 2 h glucose and insulin concentrations (ISI&lt;sup&gt;°&lt;/sup&gt;)</td>
<td>→</td>
</tr>
<tr>
<td>Tighe et al. 2010</td>
<td>102 F, 104 M healthy subjects/MetS (52 ± 1 y)&lt;br&gt;BMI 28 ± 0.5 kg/m²</td>
<td>Randomized parallel, 16 wk (3 treatment groups)</td>
<td>Group 1: whole wheat foods, 3 servings (70-80 g wholemeal bread and 30-40 g WG cereals)&lt;br&gt;Group 2: whole wheat foods, 1 serving, and oats, 2 servings</td>
<td>Refined grain foods</td>
<td>Fasting insulin and glucose concentrations (HOMA-IR&lt;sup&gt;°&lt;/sup&gt;, Modified QUICKI&lt;sup&gt;°&lt;/sup&gt;)</td>
<td>→</td>
</tr>
<tr>
<td>Landberg et al. 2010</td>
<td>17 M with prostate cancer (73.5 ± 4.6 y)&lt;br&gt;BMI 27.5 ± 4.6 kg/m²</td>
<td>Randomized crossover 2 x 6 wk separated a by 2 wk washout</td>
<td>Wholegrain rye and rye bran products: 247 ± 34 g bread, 89 ± 17 g crisp bread, 50 ± 9 g breakfast cereals, 35 ± 10 g porridge (uncooked)&lt;br&gt;Fibre intake from the test products: 58 ± 7 g</td>
<td>Refined wheat grain products with added cellulose: 245 ± 39 g bread, 96 ± 19 g crisp bread, 39 ± 15 g breakfast cereals, 29 ± 5 g porridge (uncooked)&lt;br&gt;Fibre intake from the test products: 57 ± 9 g</td>
<td>Fasting insulin concentrations</td>
<td>↑</td>
</tr>
<tr>
<td>Giacco et al. 2010</td>
<td>3 F, 12 M healthy subjects (55 ± 8 y)&lt;br&gt;BMI 27 ± 3.0 kg/m²</td>
<td>Randomized crossover 2 x 3 wk&lt;br&gt;No washout</td>
<td>Wholemeal wheat bread, pasta, rusks and crackers (dietary cereal fibre content = 23 g)</td>
<td>Refined wheat bread, pasta, rusks and crackers (dietary cereal fibre content = 10 g)</td>
<td>Fasting insulin and glucose concentrations (HOMA-IR&lt;sup&gt;°&lt;/sup&gt;)</td>
<td>→</td>
</tr>
</tbody>
</table>

(Table continues on the next page)
### Appendix table (continues)

<table>
<thead>
<tr>
<th>Reference</th>
<th>Characteristics of participants</th>
<th>Study design</th>
<th>Wholegrain products (intake/d)</th>
<th>Control products (intake/d)</th>
<th>Methods for the evaluation of insulin sensitivity</th>
<th>Insulin sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brownlee et al. 2010</td>
<td>216 healthy subjects no WG consumer (age 46 ± 10 y) BMI 30 ± 4.25 kg/m²</td>
<td>Randomized parallel, 16 wk Treatment groups: Group 1: WG 60 g/d for 16 weeks; Group 2: WG 60 g/d for 8 weeks followed by 120 g/d until the end of intervention. Group 3: RG</td>
<td>Subjects freely selected from the provided foods: Whole wheat bread, shredded wheat, cheerios, porridge oats, brown basmati rice, whole wheat pasta, weetabix, oat bar, WG crisps In all products content of WG was &gt; 50 % except rice and pasta (Intake of WG ingredients was 74 ± 28.5 g in the group 1 and 115 ± 39.6 g in the group 2)</td>
<td>Refined grain foods (no dietary change)</td>
<td>Fasting insulin and glucose concentrations (modified QUICKI)</td>
<td>→</td>
</tr>
</tbody>
</table>

WG = wholegrain; RG = refined grains; FSIGT = frequently sampled intravenous glucose tolerance test; † improved; → no effect; wk = week. Food intake expressed as mean ± SD. MetS = metabolic syndrome

*a Insulin sensitivity index

*b Homeostatic model assessment

*c Quantitative insulin sensitivity check index
APPENDIX 2. Study III.

Supplemental table 1. Description of food groups

<table>
<thead>
<tr>
<th>Food group</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wholegrain breads</td>
<td>Containing &gt; 50 % whole grain ingredients by weight</td>
</tr>
<tr>
<td>High-fibre breads (other than</td>
<td>Containing ≥ 6 % fiber</td>
</tr>
<tr>
<td>wholegrain breads)</td>
<td></td>
</tr>
<tr>
<td>Refined white bread</td>
<td>Containing &lt; 6 % fiber</td>
</tr>
<tr>
<td>Endosperm rye bread</td>
<td>Endosperm rye flour used as sole flour in baking</td>
</tr>
<tr>
<td>Wholemeal pasta</td>
<td></td>
</tr>
<tr>
<td>Other pasta</td>
<td>Elavena biscuits (Raisio plc, Finland) containing 26-36 % oat</td>
</tr>
<tr>
<td>Oat biscuit</td>
<td></td>
</tr>
<tr>
<td>Other wholegrain products</td>
<td>Such as porridges, muesli, whole grain rice, rye flour</td>
</tr>
<tr>
<td>Other grain products</td>
<td></td>
</tr>
<tr>
<td>Bran and germ</td>
<td>Such as rice, corn, low fiber cereals and muesli, pastries</td>
</tr>
<tr>
<td>Potatoes</td>
<td></td>
</tr>
<tr>
<td>Root vegetables</td>
<td></td>
</tr>
<tr>
<td>Vegetables</td>
<td>Fresh, frozen, and canned</td>
</tr>
<tr>
<td>Pulses</td>
<td></td>
</tr>
<tr>
<td>Nuts and seeds</td>
<td></td>
</tr>
<tr>
<td>Fruits</td>
<td></td>
</tr>
<tr>
<td>Berries</td>
<td></td>
</tr>
<tr>
<td>Jam</td>
<td></td>
</tr>
<tr>
<td>Fruit and berry juices</td>
<td></td>
</tr>
<tr>
<td>Butter</td>
<td></td>
</tr>
<tr>
<td>Spreads</td>
<td></td>
</tr>
<tr>
<td>Margarine</td>
<td>≥ 60 % and &lt; 60 % fat containing margarines combined</td>
</tr>
<tr>
<td>Oil</td>
<td></td>
</tr>
<tr>
<td>Low fat milk, and sour milk</td>
<td>&lt; 1 % fat</td>
</tr>
<tr>
<td>Milk and sour milk</td>
<td>≥ 1 % fat</td>
</tr>
<tr>
<td>Sour milk products</td>
<td>Yogurt, curdled milk, quark</td>
</tr>
<tr>
<td>Cheese</td>
<td></td>
</tr>
<tr>
<td>Cream</td>
<td>Includes cream and ice cream</td>
</tr>
<tr>
<td>Red meat</td>
<td>Pork and beef</td>
</tr>
<tr>
<td>Poultry</td>
<td></td>
</tr>
<tr>
<td>Sausages</td>
<td></td>
</tr>
<tr>
<td>Cold cuts</td>
<td></td>
</tr>
<tr>
<td>Fish</td>
<td></td>
</tr>
<tr>
<td>Egg</td>
<td></td>
</tr>
<tr>
<td>Coffee</td>
<td></td>
</tr>
<tr>
<td>Tea</td>
<td></td>
</tr>
<tr>
<td>Alcoholic drinks</td>
<td></td>
</tr>
<tr>
<td>Sugar</td>
<td></td>
</tr>
<tr>
<td>Sweets and chocolate</td>
<td></td>
</tr>
</tbody>
</table>
APPENDIX 3. Study III.

Supplemental table 2. Intake of non-grain foods at wk 0 and 11 in RB and WWB groups1, 2

<table>
<thead>
<tr>
<th></th>
<th>RB group (n=27)</th>
<th>WWB group (n=24)</th>
<th>P3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>wk 0</td>
<td>wk 11</td>
<td>wk 0</td>
</tr>
<tr>
<td>Potatoes</td>
<td>77 (22-208)</td>
<td>85 (0-231)</td>
<td>77 (0-233)</td>
</tr>
<tr>
<td>Root vegetables</td>
<td>15 (0-129)</td>
<td>8 (0-112)</td>
<td>26 (0-86)</td>
</tr>
<tr>
<td>Vegetables</td>
<td>114 (6-372)</td>
<td>119 (12-310)</td>
<td>106 (7-291)</td>
</tr>
<tr>
<td>Fruits</td>
<td>79 (0-594)</td>
<td>85 (0-280)</td>
<td>87 (0-319)</td>
</tr>
<tr>
<td>Berries</td>
<td>12 (0-75)</td>
<td>6 (0-305)</td>
<td>10 (0-55)</td>
</tr>
<tr>
<td>Fruit and berry juices</td>
<td>21 (10-1063)</td>
<td>52 (0-825)</td>
<td>50 (0-600)</td>
</tr>
<tr>
<td>Spreads</td>
<td>7 (0-70)</td>
<td>4 (0-66)</td>
<td>10 (2-45)</td>
</tr>
<tr>
<td>Margarine</td>
<td>11 (0-46)</td>
<td>21 (0-64)</td>
<td>13 (0-106)</td>
</tr>
<tr>
<td>Low fat milk and sour milk</td>
<td>273 (0-667)</td>
<td>200 (0-688)</td>
<td>147 (0-901)</td>
</tr>
<tr>
<td>Milk and sour milk</td>
<td>77 (3-264)</td>
<td>28 (0-539)</td>
<td>83 (4-1051)</td>
</tr>
<tr>
<td>Sour milk products</td>
<td>46 (0-250)</td>
<td>25 (0-206)</td>
<td>50 (0-400)</td>
</tr>
<tr>
<td>Cheese</td>
<td>25 (0-157)</td>
<td>28 (0-137)</td>
<td>29 (0-104)</td>
</tr>
<tr>
<td>Cream</td>
<td>13 (0-220)</td>
<td>15 (0-61)</td>
<td>16 (0-135)</td>
</tr>
<tr>
<td>Red meat</td>
<td>35 (0-296)</td>
<td>59 (0-485)</td>
<td>40 (0-167)</td>
</tr>
<tr>
<td>Poultry</td>
<td>0 (0-150)</td>
<td>0 (0-100)</td>
<td>0 (0-113)</td>
</tr>
<tr>
<td>Sausages</td>
<td>19 (0-63)</td>
<td>31 (0-129)</td>
<td>13 (0-102)</td>
</tr>
<tr>
<td>Cold cuts</td>
<td>8 (0-74)</td>
<td>10 (0-63)</td>
<td>10 (0-65)</td>
</tr>
<tr>
<td>Fish</td>
<td>32 (0-234)</td>
<td>25 (0-180)</td>
<td>23 (0-174)</td>
</tr>
<tr>
<td>Egg</td>
<td>14 (0-61)</td>
<td>10 (0-67)</td>
<td>15 (0-80)</td>
</tr>
<tr>
<td>Coffee</td>
<td>403 (0-1276)</td>
<td>450 (0-863)</td>
<td>450 (100-950)</td>
</tr>
<tr>
<td>Tea</td>
<td>0 (0-725)</td>
<td>0 (0-888)</td>
<td>56 (0-475)</td>
</tr>
<tr>
<td>Alcoholic drinks</td>
<td>0 (0-495)</td>
<td>0 (0-1073)</td>
<td>0 (0-1095)</td>
</tr>
<tr>
<td>Sugar</td>
<td>9 (0-36)</td>
<td>6 (0-42)</td>
<td>10 (1-46)</td>
</tr>
</tbody>
</table>

1Values are median (minimum-maximum)
2Intakes of bran and germ, pulses, nuts and seeds, jam, butter, oil, and sweets and chocolate were very low among the participants and are not reported. Different from wk 0: 3p<0.05 (pairwise t-test with FDR correction). RB, rye bread; WWB, white wheat bread.
3p-value for group x time interaction (linear mixed model with FDR correction)
### APPENDIX 4. Study III.

**Supplemental table 3.** Bacterial phylotypes with altered abundance in the WWB group over the intervention\(^1\)

<table>
<thead>
<tr>
<th>Phylum/Order</th>
<th>Genus-like</th>
<th>Phylotype</th>
<th>Log fold change</th>
<th>p-value (^2)</th>
<th>Changed phylotypes per genus-like group (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actinobacteria</td>
<td>Atopobium*</td>
<td>Atopobium minutum</td>
<td>0.049</td>
<td>0.024</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Atopobium parvulum</td>
<td>0.056</td>
<td>0.036</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Collinsella*</td>
<td>Collinsella stercoris</td>
<td>0.048</td>
<td>0.043</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Uncultured bacterium clone Eldhufec074</td>
<td>0.248</td>
<td>0.049</td>
<td></td>
</tr>
<tr>
<td>Bacteroidetes</td>
<td>Propionibacterium</td>
<td>Propionibacterium acnes</td>
<td>0.068</td>
<td>0.018</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>Streptococcus intermedium et rel.</td>
<td>Streptococcus constellatus</td>
<td>0.094</td>
<td>0.005</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>Allistipes et rel.</td>
<td>Uncultured bacterium MG06</td>
<td>-0.118</td>
<td>0.043</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>Bacteroides plebeius et rel.*</td>
<td>Bacteroides coprocola</td>
<td>-0.190</td>
<td>0.009</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bacterium adhufec367</td>
<td>-0.139</td>
<td>0.039</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Bacteroides uniformis et rel.</td>
<td>Bacteroides uniformis</td>
<td>-0.244</td>
<td>0.036</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>Bacteroides vulgatus et rel.*</td>
<td>Uncultured bacterium ABLC8</td>
<td>-0.311</td>
<td>0.020</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Uncultured bacterium OLDA-A2</td>
<td>-0.311</td>
<td>0.020</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Uncultured bacterium ABLC36</td>
<td>-0.304</td>
<td>0.021</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Uncultured bacterium LCLC45</td>
<td>-0.304</td>
<td>0.021</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Uncultured bacterium LCLC5</td>
<td>-0.304</td>
<td>0.021</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Uncultured bacterium LCLC6</td>
<td>-0.304</td>
<td>0.021</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Uncultured bacterium LCRC22</td>
<td>-0.304</td>
<td>0.021</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bacteroides vulgatus</td>
<td>-0.255</td>
<td>0.031</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Parabacteroides distasonis et rel.</td>
<td>Parabacteroides merdae</td>
<td>-0.139</td>
<td>0.009</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Prevotella oralis et rel.</td>
<td>Prevotella oralis</td>
<td>-0.058</td>
<td>0.024</td>
<td>17</td>
</tr>
<tr>
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<td>Prevotella ruminicola et rel.</td>
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1 For each altered phylotype, taxonomic information is provided on genus-like and phylum/order level. *Statistically significantly enriched genus-like groups based on the 55 significantly changed phylotypes. WWB, white wheat bread.
2 p-value for within group comparisons (pairwise t-test with FDR correction)
Supplemental figure 1. Hierarchical clustering of the HITChip microbiota profiles among the participants (n=51). Dash lines indicate participants in the WWB group and the solid lines participants in the RB group. Numbers denote participants, and the two branches of the tree represent microbiota composition of the pre- and post-intervention samples, respectively. HITChip, Human Intestinal Tract Chip; RB, rye bread; WWB, white wheat bread.
APPENDIX 6. Study III.

Supplemental figure 2. Baseline abundance and changes of the significantly enriched genus-like groups among the participants in the WWB group (n=24). Individual participants are shown by spots. WWB, white wheat bread.
Supplemental figure 3. Change in the relative abundance of *Bacteroides vulgatus et rel.* among participants in the A) WWB group (n=24) and B) RB group (n=27) during the intervention. Group mean and 95% confidence interval are shown by the horizontal and dash lines, respectively. RB, rye bread; WWB, white wheat bread.
Supplemental figure 4. A) Bacteria contributing to separation between the post-intervention samples of the WWB group (n=24) and the pooled set of pre-intervention samples (WWB group, n=24; RB group, n=27) and RB group post-intervention samples (n=27) in bootstrap aggregated RDA. B) Bootstrapped mean loadings (first RDA axis) of the bacteria contributing to the separation between the post-intervention samples of the WWB group and the other samples in the WWB and RB groups. RB, rye bread; RDA, redundancy analysis; WWB, white wheat bread.
Supplemental figure 5. The proportion of variation (%) in bacterial genus-like groups associated with the WWB and RB groups in bootstrap aggregated PLS analysis. Only bacteria with $\geq 10\%$ of associated variation are shown. PLS, partial least squares; RB, rye bread; WWB, white wheat bread.
Jenni Lappi
Effects of Wholegrain Foods and Grain Fibre in Intestinal Tract in Relation to Glucose Metabolism
With an Emphasis on Wheat and Rye Bread Effects

This study investigated effects of wholegrain and fibre-rich wheat and rye foods in the intestinal tract influencing glucose metabolism. Effects of bread type on postprandial responses and longer-term effects of the grain foods on glucose metabolism were studied along with large-intestinal phenomena such as intestinal microbiota composition. This thesis indicated that effects of wholegrain and fibre-rich breads on glucose metabolism are mediated via acute postprandial glucose and insulin responses, rather than via the studied effects in the large intestine.