# Impact of Consumption of Beta-glucans on the Intestinal Microbiota and Glucose and Lipid Metabolism

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**Study performed by:**

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# 1. INTRODUCTION

This document is established to provide information about dietary clinical trial "Impact of Consumption of Beta-glucans on the Intestinal Microbiota and Glucose and Lipid Metabolism". Document was prepared when clinical trial was completed and contains basic information about study design, protocol and methodology used.

## 1.1. Objective

The purpose of this study was to investigate if daily consumption of barley beta-glucans effect lipid and glucose metabolism and alter intestinal microbiota composition in participants with metabolic syndrome or with high risk for metabolic syndrome development. We conducted 4-week long, placebo controlled dietary study, where participants consumed bread with (test group) or without (control group) enriched beta-glucans bread.

## 1.2. Hypothesis

1.      4- week consumption of beta-glucans alters the composition of intestinal microbiota and changes the ratio of different bacteria groups and consequently effects production of short chain fatty acids in a population with metabolic syndrome or with high risk for metabolic syndrome development.

2.      4- week consumption of beta-glucans has an influence on glucose metabolism and consequently improves insulin resistance within people with metabolic syndrome or with high risk for metabolic syndrome development.

3.      4 - week consumption of beta-glucans improves specific plasma lipid content in a population with metabolic syndrome or with high risk for metabolic syndrome development.

# 2. STUDY METHODS

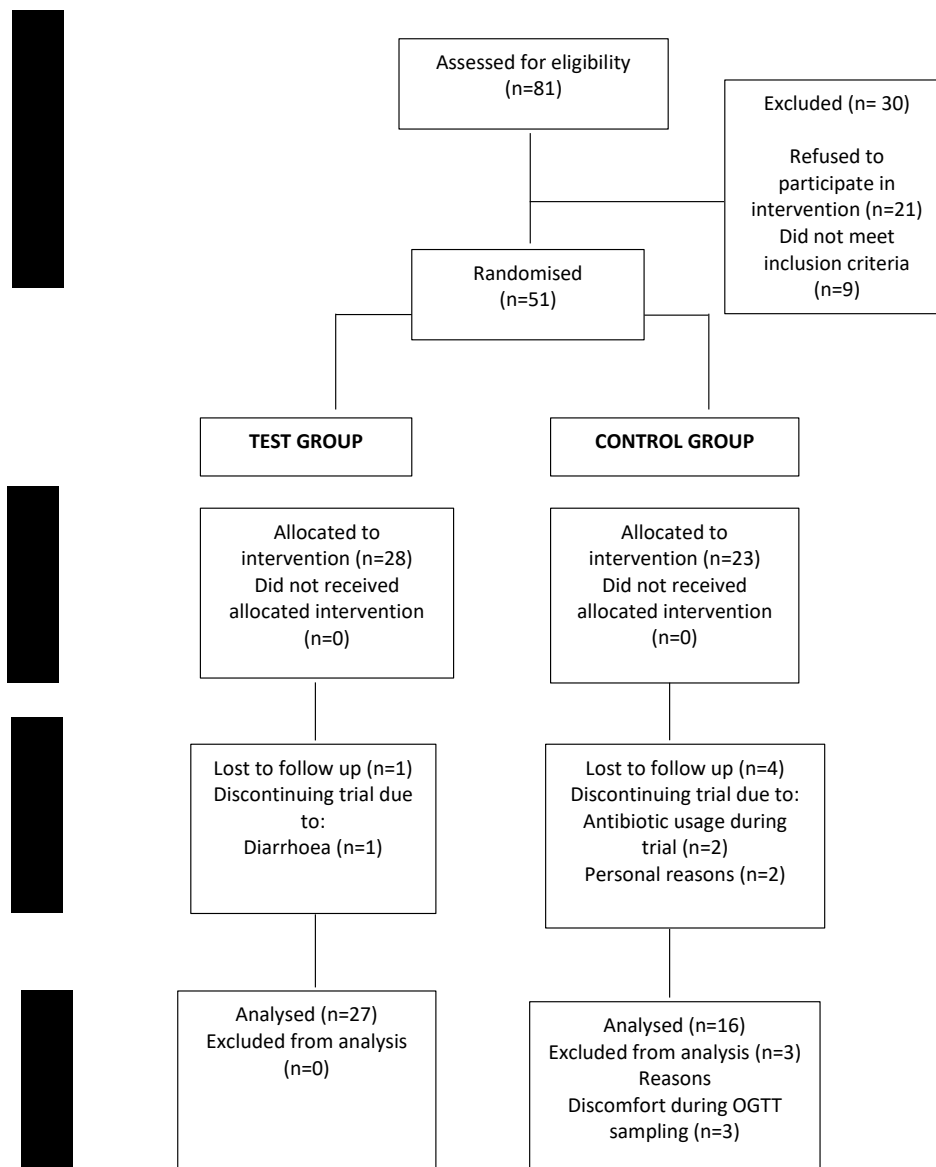## 2.1. Trial design and participants enrolment

Study was performed as double blind, randomised, placebo controlled clinical trial and was performed regarding to CONSORT 2010 recommendations for randomised clinical trials [1]. The study was approved by the Republic of Slovenia National Medical Ethics Committee (112/08/13). Participant enrolment was performed within a national programme for metabolic disorder prevention in community health centres in Slovenia in association with family medicine doctors. Participants that met including criteria and had metabolic syndrome or high risk for metabolic development were suggested to participate in clinical trial.

Table 1: **INCLUSION CRITERIA**

| Parameter | Inclusion criteria* |
|---|---|
| Age | 30 – 70 years |
| Total cholesterol levels | ≥ 5.0 mmol /L |
| Waist circumference | Women > 80 cm<br>Men > 94 cm |
| **And presence of at least one of the remaining determinants for MS** | |
| HDL-cholesterol | Women < 1.29 mmol/L<br>Men < 1.03 mmol/L |
| Raised blood pressure | Systolic blood pressure ≥ 130 mm Hg<br>Diastolic blood pressure ≥ 85 mm Hg |
| Raised fasting plasma glucose | ≥ 5.6 mmol/L |

*Participants diagnosed with metabolic syndrome and meeting the age criteria were directly included in the study. Exclusion criteria were diabetes type II, thyroid disorders, kidney disorders and antibiotic treatment.

Participants who met the inclusion criteria started the intervention after agreement with study conditions and procedures. Participants that agreed to participate were randomised and enrolled in trial as the control or test group (Figure 1).

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                    ┌─────────────────────┐
                    │ Assessed for        │       ┌──────────────────────┐
                    │ eligibility         │       │ Excluded (n= 30)     │
                    │ (n=81)              │       │                      │
                    └─────────────────────┘       │ Refused to           │
                              │                    │ participate in       │
                              │                    │ intervention (n=21)  │
                              │                    │ Did not meet         │
                    ┌─────────────────────┐       │ inclusion criteria   │
                    │ Randomised          │       │ (n=9)                │
                    │ (n=51)              │       └──────────────────────┘
                    └─────────────────────┘
                     │                  │
          ┌────────────────┐   ┌────────────────┐
          │ TEST GROUP     │   │ CONTROL GROUP  │
          └────────────────┘   └────────────────┘
                 │                    │
   ┌────────────────────┐   ┌────────────────────┐
   │ Allocated to       │   │ Allocated to       │
   │ intervention (n=28)│   │ intervention (n=23)│
   │ Did not received   │   │ Did not received   │
   │ allocated          │   │ allocated          │
   │ intervention (n=0) │   │ intervention (n=0) │
   └────────────────────┘   └────────────────────┘
            │                        │
   ┌────────────────────┐   ┌────────────────────┐
   │ Lost to follow up  │   │ Lost to follow up  │
   │ (n=1)              │   │ (n=4)              │
   │ Discontinuing trial│   │ Discontinuing trial│
   │ due to:            │   │ due to:            │
   │ Diarrhoea (n=1)    │   │ Antibiotic usage   │
   │                    │   │ during trial (n=2) │
   │                    │   │ Personal reasons   │
   │                    │   │ (n=2)              │
   └────────────────────┘   └────────────────────┘
            │                        │
   ┌────────────────────┐   ┌────────────────────┐
   │ Analysed (n=27)    │   │ Analysed (n=16)    │
   │ Excluded from      │   │ Excluded from      │
   │ analysis (n=0)     │   │ analysis (n=3)     │
   │                    │   │ Reasons            │
   │                    │   │ Discomfort during  │
   │                    │   │ OGTT sampling (n=3)│
   └────────────────────┘   └────────────────────┘
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**Figure 1: Participants flow diagram.**

## 2.2. Dietary intervention

Participants consumed bread with beta-glucans content of approximately 3,4 g beta-glucans per 100 g of bread in 4-week long intervention. Before study participants had two-week washout period without consuming any pre- and pro-biotics. Participants retained their usual eating habits during the

intervention. A 72-hour dietary recall was performed in order to control participants' diet and eating habits. Participants were told to consume 200 g of bread daily, not knowing whether the bread was experimental or control. Participants included in the test group consumed 200 g of BGB, containing more than 6 g of barley beta glucans, daily. Participants in the control group consumed 200 g of CWB without added beta glucans. 72-hour dietary recall was performed before intervention and at the end of the trial to evaluate daily nutrient and energetic input of each participant.

## 2.3. Primary outcome measures

Participants gave blood and stool samples a day before intervention with barley beta-glucans. Blood sampling was performed for insulin resistance determination and for lipid profile determination (Total cholesterol, HDL-cholesterol, LDL-cholesterol, triglycerides). They also had option to give a blood sample for genetic investigation of genes associated with lipid metabolism (apoE).

Analysis were performed in accordance with health community centres in Ajdovščina and Ljubljana, Slovenia. Venous blood samples were obtained by professional medicine personnel for analysis of fasting glucose, lipids and insulin.

### 2.3.1. Lipid profile

All blood lipid measurements were determined with standard diagnostic methods (Roche/Hitachi Cobas c system). Total cholesterol was determined with an enzymatic method using cholesterol esterase, cholesterol oxidase and peroxidase [2]. Blood triglycerides were determined with a four-step enzymatic method involving lipoprotein lipase, glycerol kinase, L-α-glycerol phosphate oxidase and NAD-peroxidase [3,4]. LDL-cholesterol was determined with cholesterol oxidase and peroxidase [5]. HDL-cholesterol was determined with cholesterol esterase and cholesterol oxidase [6].

### 2.3.2. Oral glucose tolerance test (OGTT)

OGTT was performed one day before and one day after the four-week intervention [7]. Participants fasted overnight (for at least 10 hrs). Fasting blood samples were obtained for analysis of baseline glucose and insulin levels. After the consumption of glucose solution (75 g of glucose dissolved in 250 ml of water) in five minutes time, blood samples were taken, at 30, 60 and 120 minutes. Concentrations of blood glucose were determined with a standardised spectrophotometric methodology (Roche Hitachi Cobas c system (GLUC3)). The procedure is based on hexokinase and glucose-6-phosphate dehydrogenase reaction [8]. Blood insulin was determined by immunoradiometric assay (Biosource Europe S.A., Nivelles).

### 2.3.3. Faecal sampling and DNA isolation

Faecal samples were collected one day before the start and at the end of the intervention. The samples were frozen immediately and stored at -20 °C until use. DNA was isolated within one week after sampling. DNA isolation was performed in parallel using the protocol for isolation of DNA from faecal samples described by Yu and Morrison (2004) [9].

### 2.3.4. Microbial community analysis

**DGGE:** PCR-DGGE fingerprinting was performed to check the quality of isolated DNA between parallel samples and to compare the individual profiles. PCR-DGGE analysis was performed as described by Matijašić et al. (2014) [10].

**Real-time PCR analysis** was performed by an established procedure for quantification of bacteria in faecal samples [10] with slight modifications using a ViiA™ 7 Real Time PCR System (Applied Biosystems, USA). Data were analysed with ViiA™ 7 Software 1.2.2 (Applied Biosystems, USA).

**Next generation pair end sequencing**: For 16S rRNA sequencing, 23 participants were chosen randomly among those with both samples qualified for sequencing after DGGE examination. In total, 46 samples from 14 persons in the test group and nine persons in the control group were examined.

The Illumina Miseq platform with v.3 2x300 pair end technology was used for sequencing V3 and V4 16S rRNA regions (Microsynth). After demultiplexing, raw reads were processed following the UPARSE pipeline (Usearch v.8, June 2015) [11]. Stringent quality filtering based on a low expected number of errors was used for OTU formation with algorithm parameters optimized after mock sample analysis (34 strains). With relaxed quality filtered sequences, more than 91 % of raw reads could be placed into an OTU with a 0.03 distance criterion. Presumptive OTU identity was determined with the 'usearch_global' function searched against Silva NR SSU v.119 and LTP SSU type strains v.121 database. Taxonomy was assigned to OTUs with Wang et al.'s (2007) [12] method implemented in Mothur v.1.35 and the RDP training set 14 [13]. FastTree2 was used for the generation of an approximately maximum-likelihood phylogenetic tree [14]. Taxonomy, the OTU table and the phylogenetic tree were handled with the 'R' package Phyloseq for statistical analysis and graphics [15].

## 2.4. Secondary outcome measures
After 4 weeks of dietary intervention protocol of primary outcome measures was repeated.

## 2.5. Missing data
Due to complex presentation of data for changes in gut microbiota composition, we can`t provide results for primary and secondary outcome measure of microbiota composition.

# 3. STATISTICS

For analysis of participants' anthropometric and metabolic characteristics, RT-PCR results and microbial richness and diversity measures, a Wilcoxon signed-rank test or paired samples $t$-test were used as appropriate with $P < 0.05$ for significance and $P < 0.1$ for reporting a trend (SPSS v.21). Statistical sequence analysis was based on differential abundance testing using the 'R' package DESeq 2 [16]. Differential abundance that occurred in test group subjects after dietary intervention but not in the control group was determined in a model with interaction (time, group and subject). Significance was reported for $P < 0.05$ after multiple inference correction (Benjamini-Hochberg), and all OTUs with $P < 0.05$ before correction were screened for trends. Initial microbial abundance differences between test group subjects who responded to dietary intervention with a 10 % plasma total cholesterol concentration drop and those without reduction were also tested with Deseq2 [16]. OTUs with $P < 0.1$ after correction were considered significant. Alpha and beta diversity measures and PCoA of weighted UniFrac distances were calculated and plotted with Phyloseq [15].

# 3. REFERENCES

[1]     Schulz KF, Altman DG, Moher D. CONSORT 2010 statement: Updated guidelines for reporting parallel group randomised trials. PLoS Med 2010;7. doi:10.1371/journal.pmed.1000251.

[2]     Allain CC, Poon LS, Chan CSG, Richmond W, Fu PC. Enzymatic Determination of Total Serum Cholesterol. Clin Chem 1974;20:470–5.

[3]     Fossati P, Prencipe L. Serum triglycerides determined colorimetrically with an enzyme that produces hydrogen peroxide. Clin Chem 1982;28:2077–80.

[4]     McGowan MW, Artiss JD, Strandbergh DR, Zak B. A peroxidase-coupled method for the colorimetric determination of serum triglycerides. Clin Chem 1983;29:538–42.

[5]     Sugiuchi H, Irie T, Uji Y, Ueno T, Chaen T, Uekama K, et al. Homogeneous assay for measuring low-density lipoprotein cholesterol in serum with triblock copolymer and alpha-cyclodextrin sulfate. Clin Chem 1998;44:522–31.

[6]     Sugiuchi H, Uji Y, Okabe H, Irie T, Uekama K, Kayahara N, et al. Direct measurement of high-

density lipoprotein cholesterol in serum with polyethylene glycol-modified enzymes and sulfated Alpha-cyclodextrin. Clin Chem 1995;41:717–23.

[7]     Diabetes mellitus: Report of a WHO study group. 1985.

[8]     Wu AHB. Tietz clinical guide to laboratory tests. 4th ed. Missouri: W.B.Saunders Company; 2006.

[9]     Yu Z, Morrison M. Improved extraction of PCR-quality community DNA from digesta and fecal samples. Biotechniques 2004;36:808–12.

[10]    Matijašić BB, Obermajer T, Lipoglavšek L, Grabnar I, Avguštin G, Rogelj I. Association of dietary type with fecal microbiota in vegetarians and omnivores in Slovenia. Eur J Nutr 2014;53:1051–64. doi:10.1007/s00394-013-0607-6.

[11]    Edgar RC. UPARSE: Highly accurate OTU sequences from microbial amplicon reads. Nat Methods 2013;10:996–8. doi:10.1038/nmeth.2604.

[12]    Wang Q, Garrity GM, Tiedje JM, Cole JR. Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl Environ Microbiol 2007;73:5261–7. doi:10.1128/AEM.00062-07.

[13]    Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, et al. Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol 2009;75:7537–41. doi:10.1128/AEM.01541-09.

[14]    Price MN, Dehal PS, Arkin AP. FastTree 2 - Approximately maximum-likelihood trees for large alignments. PLoS One 2010;5. doi:10.1371/journal.pone.0009490.

[15]    McMurdie PJ, Holmes S. Phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. PLoS One 2013;8. doi:10.1371/journal.pone.0061217.

[16]    Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol 2014;15. doi:10.1186/s13059-014-0550-8.