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RESEARCH ARTICLE

Effects of *in vitro* fermentation of barley β-glucan and sugar beet pectin using human fecal inocula on cytokine expression by dendritic cells

Christiane Rösch[1,3], Nico Taverne[2], Koen Venema[3,4], Harry Gruppen[1], Jerry M. Wells[2] and Henk A. Schols[1,3]

[1] Laboratory of Food Chemistry, Wageningen University, Wageningen, the Netherlands
[2] Host Microbe Interactomics, Wageningen University, Wageningen, the Netherlands
[3] Top Institute Food and Nutrition, Wageningen, the Netherlands
[4] Beneficial Microbes Consultancy, Wageningen, the Netherlands

Scope: This study simulates the fermentation process of barley β-glucan and sugar beet pectin in the human colon and monitors the degradation products formed. Additionally, immune effects of the degradation products were investigated.

Methods and results: Immunostimulatory activity of fermentation digesta was investigated using bone marrow derived dendritic cells (BMDCs) from toll-like receptor 2/4 (TLR2/4) knockout mice, which were unresponsive to microbe-associated molecular patterns. Cytokine responses were elicited to dietary fibers and not to the SCFA and microbiota. The fermentation digesta were analyzed for their SCFA profiles and glycan metabolites over time. During fermentation the amount of insoluble precipitating fibers increased and induced as well as soluble molecules of lower molecular mass greater amounts of cytokines in BMDCs than the parental fiber. Additionally, high amounts of cytokines can be attributed to soluble galactose-rich beet pectin molecules.

Conclusions: The fermentation of the two fibers led to fiber-specific amounts of SCFA, glycosidic metabolites, and different immunomodulatory properties. BMDC from TLR2/4 knockout mice did not respond to the digest microbiota and SCFA, making it a useful approach to study temporal effects of fermentation on the immunomodulatory effects of fibers.

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1 Introduction

Barley β(1,3)(1,4)glucan and sugar beet pectin (SBP) are dietary fibers. Thus, they are indigestible in the human small intestine and end up in the large intestine, where they are fermented by the microbiota [1]. Many fermentation studies of dietary fibers have investigated their influence on microbiota composition, possible prebiotic effects and production of SCFA [2, 3]. The microbiota and SCFA are known to influence epithelial functions and immune regulation in the large bowel [4, 5]. Cryptopatches and isolated lymphoid follicles in the colon have been reported to contain M cells, which may transport particulate antigens from the lumen to dendritic cells (DCs), macrophages, and lymphocytes in the lymphoid follicles underlying the follicular epithelium [6].

Correspondence: Prof. Henk A. Schols
E-mail: henk.schols@wur.nl

Abbreviations: **BMDC**, bone marrow derived DC; **DC**, dendritic cell; **DP**, degree of polymerization; **HPAEC**, high performance anion exchange chromatography; **HMI**, host microbiota interaction; **HPSEC**, high performance size exclusion chromatography; **IMW**, intermediate molecular weight; **KO**, knock out; **LMW**, low molecular weight; **OS**, oligosaccharide; **PS**, polysaccharide; **RG**, rhamnogalacturonan; **RT**, room temperature; **SBP**, sugar beet pectin; **SHIME**, simulator of the human intestinal microbial ecosystem; **TLR**, toll-like receptor; **TNF**, tumor necrosis factor

Dietary β(1,3)-linked glucans found in cell walls of mushrooms and other fungi can elicit immune responses though binding of the β(1,3)-linked glucans to dectin-1, a member of the C-type lectin receptor family [7, 8]. Expression of the C-type lectin receptor dectin-1 was recently detected in human colonic intestinal epithelial cells [9] and was shown to be expressed in primary colonic enterocytes and induce chemokine secretion upon ligand binding to β(1,3)-linked glucans [10].

Barley-derived β-glucan is a linear polysaccharide (PS) consisting of β(1,3)- and (1,4)-linked glucose units. SBP is a much more complex fiber consisting of a homogalacturonan region that is highly methylesterified and acetylated. In addition, SBP consists of a rhamnogalacturonan region I (RG I), which is composed of alternating rhamnose and galacturonic acid units and is substituted with short neutral side chains of galactose and arabinose [11, 12].

Several *in vitro* fermentation models have been used for studies on microbiota and substrates, which all have the general limitation that they are not equipped with a mucus layer, epithelial, and other intestinal cells to take up metabolites or study effects on the host [13]. A recent development to overcome this limitation is the host microbiota interaction (HMI) model that can be connected to the simulator of the human intestinal microbial ecosystem (SHIME) fermentation model. The HMI model has a mucus layer and semipermeable layer (double functional layer), which protect the epithelial cells from the bacteria while allowing transport of low molecular weight (LMW) metabolites and oxygen diffusion [14]. When filtered yeast digesta from the SHIME fermentation model were incubated in coculture with epithelial and THP1 immune cells, proinflammatory cytokine secretion was decreased [15]. Currently, this is the only study measuring the effect of filtered fermentation digesta on cytokine responses in immune cells. However, one limitation of the HMI/SHIME model is that insoluble fiber particles that are known to be immunomodulatory [16] are excluded due to filtration.

In our *in vitro* batch fermentation, we monitor the degradation products formed during barley β-glucan and SBP fermentation using human fecal microbiota and investigate the immunomodulatory activity of complete digesta using bone marrow derived DCs (BMDCs) from toll-like receptor 2/4 (TLR2/4) knockout (KO) mice.

2 Materials and methods

2.1 Substrates

Barley β-glucan of low viscosity (Megazyme, Bray, Ireland) and a previously characterized SBP (DuPont, Brabrand, Denmark) [11], were used for the fermentation studies.

LPS from *Escherichia coli* (Sigma-Aldrich, St Louis, MO, USA) was used as a control to show unresponsiveness of the BMDCs from TLR2/4 KO mice to this common microbe-associated molecular pattern. Depleted zymosan, an insoluble β1,3-glucan from *Saccharomyces cerevisiae* (Invivogen, Toulouse, France) was used as a positive control in the immunoassays.

2.2 Culture medium for *in vitro* fermentation

The culture medium was based on the standard ileal efflux medium and modified as described elsewhere [17]. All medium components were obtained from Tritium Microbiology (Veldhoven, The Netherlands). The medium included a 1 M MES (2-(*N*-morpholino)ethanesulfonic acid hydrate) buffer to maintain pH 5.8, mimicking the pH conditions in the proximal colon [17].

2.3 Human fecal inoculum

Standardized, pooled fecal inoculum from eight healthy European adults (25–45 years old) was obtained from TNO (Zeist, the Netherlands), prepared and validated as described elsewhere [18]. The subjects were not treated with antibiotics 2 months before fecal donation. The fecal inoculum was activated in standard ileal efflux medium culture medium for 16 h and ten times with medium before use.

2.4 *In vitro* fermentation

For each time point, two 20 mL fermentation bottles were filled with 9 mL culture medium, 100 mg barley β-glucan, and 1 mL of the activated diluted fecal inoculum. SBP was presolubilized in medium solution and equally portioned in the appropriate fermentation bottle and inoculated with 1 mL of activated diluted inoculum. Hence, at the start of both fermentation experiments the fecal inoculum was diluted 100 times and fiber concentrations were 10 mg/mL. The bottles were immediately closed with rubber stoppers in an anaerobic chamber, sealed with an aluminum cap and incubated at 37°C with shaking at 130 rpm for a maximum of 48 h. Blanks with either no substrate or no inoculum were prepared as controls. Samples were taken for analysis after 0, 5, 11, 24, and 48 h for barley β-glucan and 0, 6, 11, 12, 24, and 48 h for SBP. At each time point 5.5 mL of the fermentation mixture was heated for 5 min at 100°C to stop bacterial activity.

2.5 Chemical analyses

2.5.1 Constituent monosaccharide composition

SBP fermentation digesta were separated into soluble and insoluble fractions by centrifugation (10 min, RT (room temperature), 18 000 × *g*). The constituent monosaccharide

composition of the dried soluble and insoluble SBP fermentation fractions was determined in duplicate using a prehydrolysis step with 72% w/w sulfuric acid at 30°C for 1 h, followed by hydrolysis with 1 M sulfuric acid at 100°C for 3 h. The monosaccharides released, were derivatized to alditol acetates and analyzed by GC using inositol as an internal standard [19]. The presence of uronic acid in the samples was quantified by using the colorimetric m-hydroxydiphenyl assay [20] automated on a Skalar Autoanalyzer (Skalar, Breda, the Netherlands) [21].

2.5.2 Molecular weight distribution using HPSEC

The fermentation samples were centrifuged (10 min, RT, 18 000 × *g*) and the supernatant was analyzed for molecular weight distribution of soluble PS present, by high performance size exclusion chromatography (HPSEC) on an Ultimate 3000 HPLC (Dionex, Sunnyvale, CA, USA) equipped with a Shodex RI-101 refractive index detector (Showa Denko, Tokyo, Japan). Three TSK-Gel columns connected in series (4000-3000-2500 SuperAW; 150 × 6 mm) were used for the analysis. These columns were preceded by a TSK Super AW-L guard column (35 × 4.6 mm; Tosoh Bioscience, Tokyo, Japan) and covered a molecular mass range from 0 to 250 kDa. Twenty microliters samples were injected and eluted with 0.2 M $NaNO_3$, at 40°C with a flow rate of 0.6 mL/min. Pullulan molecular mass standards (Polymer Laboratories, Palo Alto, CA, USA) were used for calibration.

2.5.3 Oligosaccharide profiling with HPAEC

After centrifugation of the fermentation samples (10 min, RT, 18 000 × *g*) the supernatants were analyzed for their oligosaccharide (OS) profile using high performance anion exchange chromatography (HPAEC) on an ICS5000 system (Dionex), with a CarboPac PA-1 column (2 × 250 mm) in combination with a Carbopac PA-1 guard column (2 × 50 mm). The elution conditions for barley β-glucan digesta were the following: the flow rate was 0.3 mL/min with an eluent profile starting with 0.02 M NaOH until 13 min, then increasing to 0.1 M NaOH in 15 min. The OSs were eluted from the column using a gradient of 0–500 mM NaOAc in 1 M NaOH (B) from 15 to 45 min, followed by a gradient to 100 % B from 45 to 46 min, which was held for 7 min. After this, the column was reequilibrated with 0.1 M NaOH (A) for 3 min and 0.02 M NaOH for 20 min. The elution gradient for SBP was different in the following condition: starting with 0–36 min from 0% B to 40% B, 36–40 min at 100 % B, and 40–55 min at 100 % A. An ICS5000 ED (Dionex) pulsed amperometric detector and Chromeleon software version 7 were used for detection and analysis.

2.5.4 SCFA

SCFAs were analyzed on a TRACE GC Ultra Gas Chromatograph system coupled with a FID detector (Interscience, Breda, the Netherlands), as described elsewhere [22]. Briefly, 50 μL of samples or standards were mixed with 50 μL of 0.15 M oxalic acid, after 30 min 150 μL water was added and 1 μL of the sample was analyzed.

2.6 Generation and stimulation of BMDCs

To obtain BMDCs, 6- to 10-week-old C57bl/6 mice lacking both TLR2 and TLR4 were euthanized and the femurs isolated, washed and gently crushed in 10 mL of RPMI-1640 medium (without HEPES) supplemented with 100 units/mL penicillin G (Invitrogen, Breda, the Netherlands) and 100 μg/mL streptomycin (Invitrogen). BM cells were filtered using a Steriflip Filter Unit (Millipore, Billerica, MA, USA) and around 2–4 × 10^4 cells per well were seeded in a 96-well flat bottom plate in complete media (RPMI-1640 medium containing 10% heat-inactivated fetal calf serum (Sigma-Aldrich), 100 units/mL penicillin G, 100 μg/mL streptomycin, 20 ng/mL of recombinant mouse granulocyte-macrophage colony-stimulating-factor (R&D systems, Minneapolis, MN, USA) and 0.05 mM of β-mercaptoethanol). Cells were incubated at 37°C in 5% v/v CO_2 atmosphere and the medium was changed every 3 days. On day 6, BMDCs were stimulated with 400 μg/mL fiber, freeze-dried digesta, or digesta blanks containing microbiota and fermentation medium. Other blanks were SCFA (acetic-, propionic-, butyric acid all in 0.1, 0.5, and 1 mg/mL concentrations in MES buffer), LPS (1 μg/mL), depleted zymosan (20 μg/mL), or medium.

2.7 Cytokine quantification in culture supernatants

Supernatants from the BMDC stimulation assays were collected after stimulation for 24 h. They were analyzed for the presence of cytokines (IL-6, IL-10, monocyte chemoattractant protein (MCP) I and tumor necrosis factor (TNF)) using a cytometric bead based BD mouse inflammation kit that enables multiplex measurements to be made in the same sample (BD Biosciences, Breda, the Netherlands) [23]. The sensitivity LODs, as described by the manufacturer, were as follows: 5.0, 17.5, 52.7, and 7.3 pg/mL for IL-6, IL-10, MCP-I, and TNF, respectively. In the mouse TNF is a synonym for TNF-α. The flow cytometry data were analyzed using the BD FCAP software (BD Biosciences).

2.8 Statistics

All statistical tests were performed using Prism 5.0 software (GraphPad, San Diego, CA, USA). Data shown are the

means and the SEM. Data were tested for normality with the D'Agostino and Pearson normality test. Statistical analyses of normally distributed data were performed with the two tailed unpaired *T*-test. Data that did not show a normal distribution were analyzed with the Welch's correction to determine equal variances between the groups. When the variances were unequal between the groups, the data were analyzed using the unpaired *T*-test with Welch's correction. Differences were considered statistically significant, highly significant and extremely significant when the p value were <0.05, <0.001, or <0.0001, respectively.

3 Results and discussion

3.1 Physicochemical and immunological characteristics of barley β-glucan and SBP

The monosaccharide composition showed that barley β-glucan consists of 99 mol% glucose. SBP consists for 80 mol% of galacturonic acid forming the pectin backbone next to small amounts of rhamnose (2 mol%), arabinose (6 mol%) and galactose (12 mol%). Of all galacturonic acid residues 53 mol % are methylesterified and 17 mol% acetylated [11]. The protein content of β-glucan and SBP was <1 and 4.8 w/w%, respectively. At RT 25 w/w% of β-glucan and 37 w/w% of SBP was water soluble (no further data shown).

In previous studies we have shown that the barley β-glucan and SBP used in this study, induced different amounts of cytokines in BMDCs from TLR2/4 KO mice, depending on fractions used and the treatments (Supporting Information Fig. 1). The parental suspensions and particulate fractions were more immunostimulatory than the soluble fractions. Heating the suspended substrates at 80°C for 10 min completely dissolved the substrates, resulting in a reduction in cytokine secretion by stimulated BMDCs. Freeze drying and resuspending the solubilized material increased the cytokine production of stimulated BMDCs (Supporting Information Fig. 1). This indicates that the immunostimulatory activity of the two pure substrates was partly affected by freeze drying and heating, which was used to stop enzymatic reactions in samples of the fermentation digesta.

3.2 pH monitoring of *in vitro* batch fermentation of fibers

The pH of the barley β-glucan *in vitro* batch fermentation started to increase from 5.8 to 6.2 within 8 h of fermentation. This may indicate the microbial utilization of protein in the medium at the beginning of the fermentation, due to a lack in degradability of the barley β-glucans, resulting in the production of ammonia instead of SCFA [24]. The pH then fluctuated to 5.9 (10 h), 6.2 (11 h), 6.3 (24 h), and 6.0 (48 h). These minimal pH changes over time suggest the presence of different glucan populations of different fermentability.

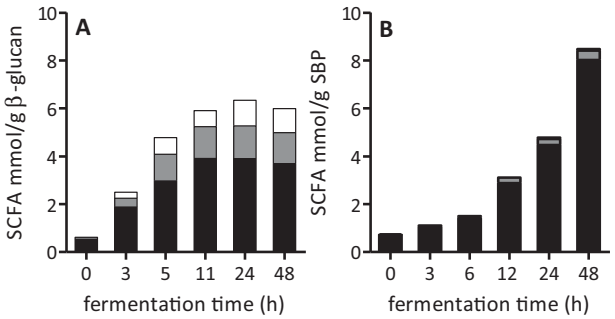

Figure 1. Levels of SCFA produced during the fermentation of barley β-glucan (A) and SBP (B) using human fecal inocula. Acetic acid (black), propionic acid (gray), and butyric acid (white).

The pH of the SBP fermentation increased from 5.8 to 6.0 within 9 h of fermentation and can be accounted for the microbial utilization of proteins in the medium as described above (Fig. 1). From 12 to 48 h of fermentation a decrease to a pH of 5.5 was observed, due to a relatively high increase in acids (SCFA and nonmethyl esterified galacturonic acid residues) (see below).

3.2.1 SCFA production

The microbial production of SCFAs during *in vitro* fermentation is one of the indicators of fermentability of the fiber and is discussed separately for each fiber.

3.2.1.1 Barley β-glucan fermentation

After 3 h fermentation the production of SCFA started to increase from 2.5 mmol/ g barley β-glucans to a maximum of 6.3 mmol/ g barley β-glucans at 24 h, of which 1.4 and 1.1 mmol/ g barley β-glucans were propionic and butyric acid, respectively (Fig. 1A). Together they constitute ca. 30 mmol% of the total SCFA from 3 h onwards. The remaining part (70 mmol%) was acetic acid, reaching a maximum of 3.9 mmol/g barley β-glucans at 24 h and remaining constant till 48 h. The molecular ratio of acetic, propionic and butyric acid at 48 h was 67:21:12.

3.2.1.2 SBP fermentation

The SCFA production of SBP seems to be in exponential increase and within 12 h fermentation ca. 40 % of the total SCFA present at 48 h are produced (Fig. 1B). The fermentation of SBP led to minor amounts of propionic acid (maximum 5 mmol% of total SCFA) and butyric acid (1 mmol% of the total SCFA) at 48 h. The molecular ratio of acetic, propionic, and butyric acid after 48 h fermentation was 95:5:1.

Overall, SBP was fermented much slower than β-glucan (Fig. 1). At 12 h SBP fermentation only 40 mmol% of total SCFA was produced, while at 11 h of β-glucan fermentation more than 90 mmol% of the total SCFA present at 48 h was

produced. The increase in SCFA for the SBP fermentation at 12 h is in line with the pH decrease from that time point onwards. This correlation between pH and SCFA produced is less pronounced due to the small pH fluctuations during the β-glucan fermentation. The total amounts of SCFA formed at the end of the fermentation (48 h) were larger for the SBP fermentation (8.5 mmol/g SBP; 6.3 mmol/g β-glucan), which is reflected in the end pH of the 48 h time points and indicates that the two substrates are fermented by the human microbiota at different rates and probably also to a different extent.

3.3 Fermentation digesta in immunoassays on BMDCs

As we showed that the two substrates were inducing cytokine secretion in BMDCs (Supporting Information Fig. 1), it was of interest to determine whether the glycosidic fermentation metabolites were also immune stimulating. The freeze-dried digesta from selected time points were tested for their capacity to elicit cytokine production in BMDCs. As was previously demonstrated (Supporting Information Fig. 3), the BMDCs from TLR2/4 KO mice were unresponsive to common bacterial molecular structures such as LPS and LTA that were anticipated to be present in the digesta [25]. Additionally, it was shown that these BMDCs were also unresponsive to flagellin (unpublished data), a component of bacterial flagella, which is normally recognized by TLR5 [26].

The high concentrations of fecal bacterial cell wall material and the possible presence of SCFA in the digesta could influence the cytokine response in these assays. To test this possibility, fermentation microbiota controls containing only the starting microbiota and fermentation medium (but no substrate) at different fermentation time points (t 0, 12, 24, 48 h) (Fig. 2) as well as different concentrations of the three main SCFA were tested for their capacity to elicit cytokine secretion (Supporting Information Fig. 2). Only low amounts of cytokines (e.g. <900 pg/mL TNF) were induced in BMDCs by the SCFA controls, which were not significant for TNF, IL-6, and IL-10 levels compared to the medium. The positive control depleted zymosan, which induces cytokines through dectin-1 signaling [27], induced still high amounts of cytokines (14192 pg/mL TNF) (Supporting Information Fig. 2). Although some fermentation microbiota blanks induced significant amounts of cytokines compared to the medium control (e.g. 3256 vs. 211 pg/mL TNF, respectively) the amounts of cytokines elicited by the digesta containing fiber were substantially greater than for the microbiota blanks (Fig. 2). It is unlikely that a change in the microbiota composition would cause such effects as the BMDCs do not respond to common microbe-associated molecular patterns found on both Gram-positive and Gram-negative bacteria (Supporting Information Fig. 3).

For the digesta containing β-glucan or SBP, all four cytokines were induced in significantly increased amounts of cytokines compared to the microbiota controls (e.g. 18 506 and 933 pg/mLTNF for β-glucan 11 h and control 0 h, respectively) (Fig. 2). This demonstrates that the cytokines were induced by the PSs present in the fermentation digesta (Fig. 2 and Supporting Information Fig. 2). For both substrates a continuous increase of all four cytokines over fermentation time with maxima at 11 h for β-glucan and 24 h for SBP fermentation, was observed. This showed that the fermentation degradation products are more immunomodulatory than the parental fiber at 0 h. Furthermore, the digesta were not affected by sample preparation steps such as heating or freeze drying as described above for the pure fibers. This was probably due to the high salt concentration of the fermentation digesta. This allowed us to draw conclusions about the immunomodulatory capacity of the fiber components present in the digesta at specific time points and will be discussed in more detail.

Our approach to study immunomodulatory effects of fermentation digesta with TLR2/4 KO mice is highly relevant to studies of immunomodulation by fibers because in the healthy intestine the mucus barrier spatially compartmentalizes bacteria to the luminal compartment of small and large intestine, limiting the induction of innate responses in the epithelium by microbes [28–30]. Additionally, the normal human colonic epithelial cells and lamina propria cells express low levels of TLR4 and its coreceptor MD-2 and the uninflamed epithelium is normally LPS unresponsive [31]. Fiber particles and digesta that are smaller than bacteria are likely to be able to diffuse through the mucus barrier and interact with pattern–recognition receptors such as dectin-1, which is expressed in epithelial cells [9].

3.3.1 Immunomodulatory effects of soluble and insoluble fiber

As it was shown that the intact fibers and their degradation products induced cytokines in BMDCs, the fiber products present at each time point were of interest. Previous studies on these two intact fibers showed a positive correlation between dose and amounts of induced cytokines (unpublished data). The insoluble β-glucans and SBP induced much higher amounts of cytokines than the soluble β-glucans and SBP fractions (Supporting Information Fig. 1). Hence, the amounts of soluble and insoluble dietary fiber in the digesta in relation to immunomodulation are of interest.

Due to very small sample amounts and the viscous characteristic of the β-glucan digesta, it was difficult to precisely separate the soluble and insoluble components of the β-glucan digesta, resulting in unreliable quantification of the soluble and insoluble β-glucans, using the enzyme kit (AACC Method 32-23.01, Megazyme). Hence, the immunomodulatory effects of soluble and insoluble β-glucans are not discussed further.

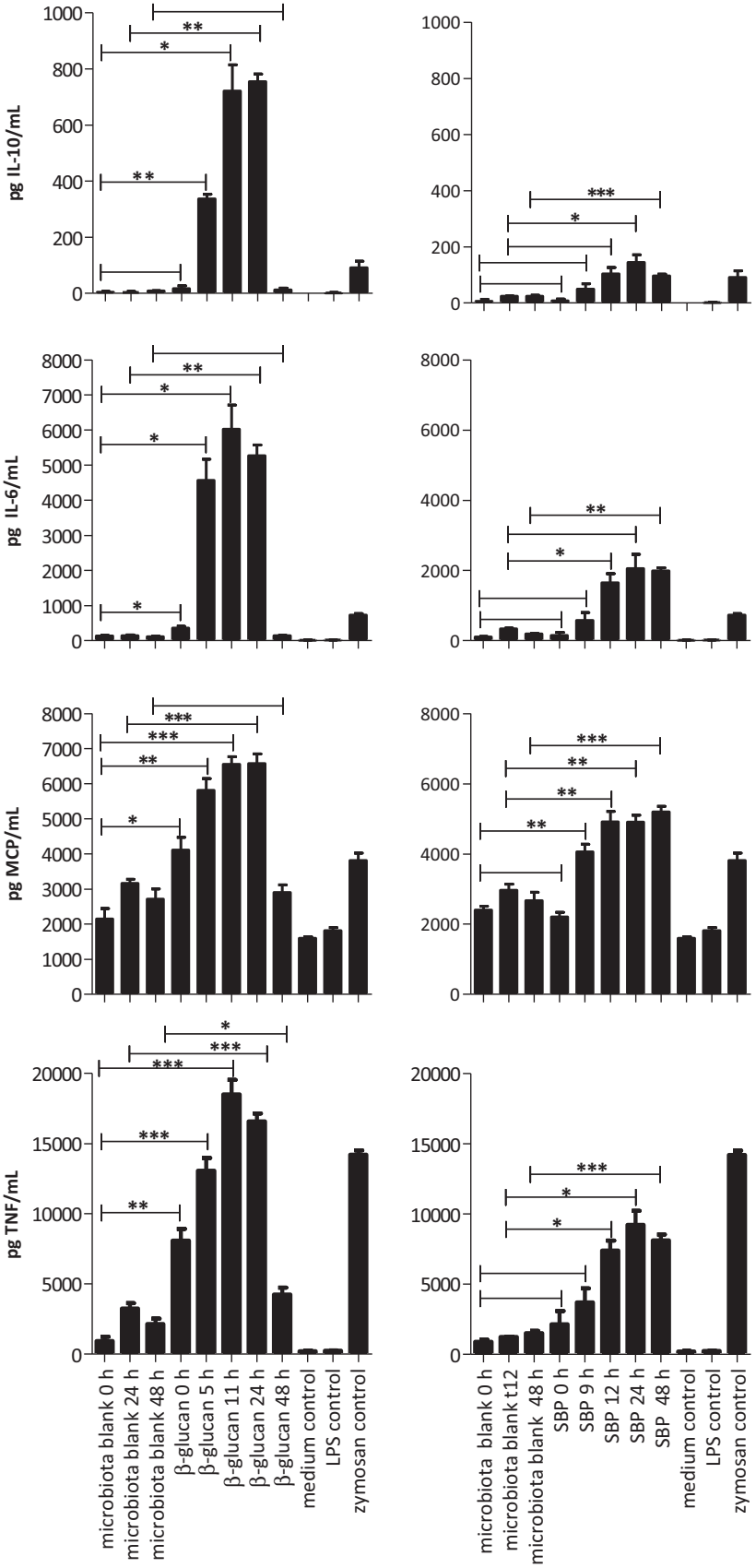

Figure 2. Induced cytokines (pg/mL) by fermentation digesta of β-glucan and SBP. Fermentation blanks contain only fermentation medium and microbiota. Stars represent classes of statistically different responses compared to the medium control (*$p < 0.05$, **$p < 0.01$, ***$p < 0.0001$).

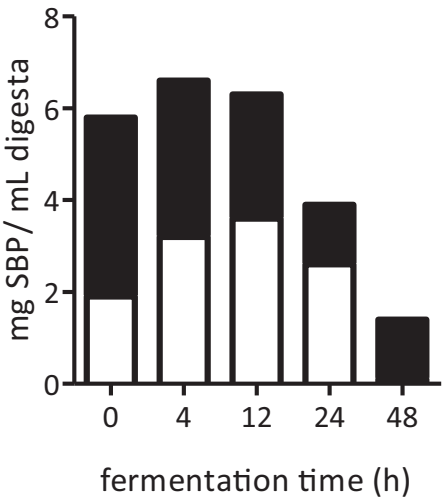

Figure 3. Amounts of soluble (black) and insoluble (white) SBP present in fermentation digesta.

3.3.1.1 SBP

For SBP the least amount of cytokines were induced at time point zero when the PS was present in its unfermented form (Fig. 2). An increase in amounts of induced cytokines was measured over time up to 24 h. The amount of insoluble SBP increased up to 12 h fermentation (Fig. 3), while the total amounts of SBP remained rather constant, indicating that precipitation of the pectin occurred. With the increased amounts of insoluble SBP, also the amounts of induced cytokines increased (Fig. 2). This suggests that the strongest cytokine responses were elicited by the insoluble SBP.

From 12 h of fermentation onwards the total amounts of SBP decreased substantially, especially the amount of soluble SBP. Additionally, the measurements of pH and amounts of SCFA decreased and increased, respectively, from 12 h onwards, indicating that the start of the SBP fermentation began at approximately 12 h. At 48 h of fermentation mainly soluble SBP was detected (1.3 mg/mL digest) and large amounts of cytokines (8112 pg/mL TNF) were induced by the digesta at this time point. This indicates that soluble glycosidic metabolites of SBP fermentation can also induce cytokine production in immune cells.

3.4 PS degradation of fibers during fermentation

As not only insoluble fibers, but also soluble fermentation metabolites seem to induce cytokines in BMDCs, the digesta were characterized for the molecular masses of the soluble PS by HPSEC (Fig. 4).

3.4.1 Molecular mass distribution during β-glucan fermentation

Soluble PSs were detected by HPSEC analysis (Fig. 4A). At time point 0 h the original molecular mass of soluble β-glucan polymer ranged between 60 and 370 kDa and remained that size within 5 h of fermentation, while the amount of these molecules decreased. This was either due to its utilization by the microbiota and/or due to modification into insoluble molecules.

At 11 h of fermentation a shift of the higher molecular mass to ca. 60 kDa was observed. Hence, endoacting enzymes, which degrade the glucan backbone, were present in the digest. At 11 h the largest amounts of cytokines were induced in the immunoassay. Within the next 13 h of fermentation the molecular mass of the soluble barley β-glucans remained the same. The amount of soluble β-glucans decreased, indicating that the glucans were partly degraded or precipitated. After 48 h the soluble polymer had almost completely disappeared.

3.4.2 Molecular mass distribution during SBP fermentation

The degradation profile of SBP revealed that mainly side chain degradation occurred, as no large shift of the molecular mass was observed during the fermentation, as was the case for the barley β-glucan fermentation.

Soluble PSs at time point 0 h consisted of a molecular mass range of 18–370 kDa. At 48 h of SBP fermentation, highest amounts of cytokines were induced and only soluble glycosidic fermentation metabolites were present (Fig. 3), having a molecular mass of 60 kDa (Fig. 4B). This indicates that soluble SBP might have been responsible for the induced cytokines.

The comparison of the two fermentation substrates showed that each dietary fiber was fermented differently, which led to different metabolites and end products. This difference was expected since the substrates are different in their chemical structure and require different enzymes for degradation. Both substrates seemed to have immunomodulatory active PSs of rather similar molecular mass (60 kDa).

3.5 OS formation during fermentation

Since soluble PSs were detected with HPSEC, the formation of LMW and intermediate molecular mass (IMW) molecules was expected. Furthermore, soluble IMW or LMW present, could be responsible for the immunomodulation of the fermentation digesta as previously suggested [32]. The formation of LMW molecules (<DP 20; DP is degree of polymerization) and IMW molecules (>DP 20; upper DP limit cannot be predicted from the pattern) during *in vitro* fermentation of the two substrates was monitored by HPAEC (Fig. 5).

3.5.1 β-Glucan fermentation degradation products

Soluble glycan degradation products formed during the barley β-glucan degradation were detected in increasing

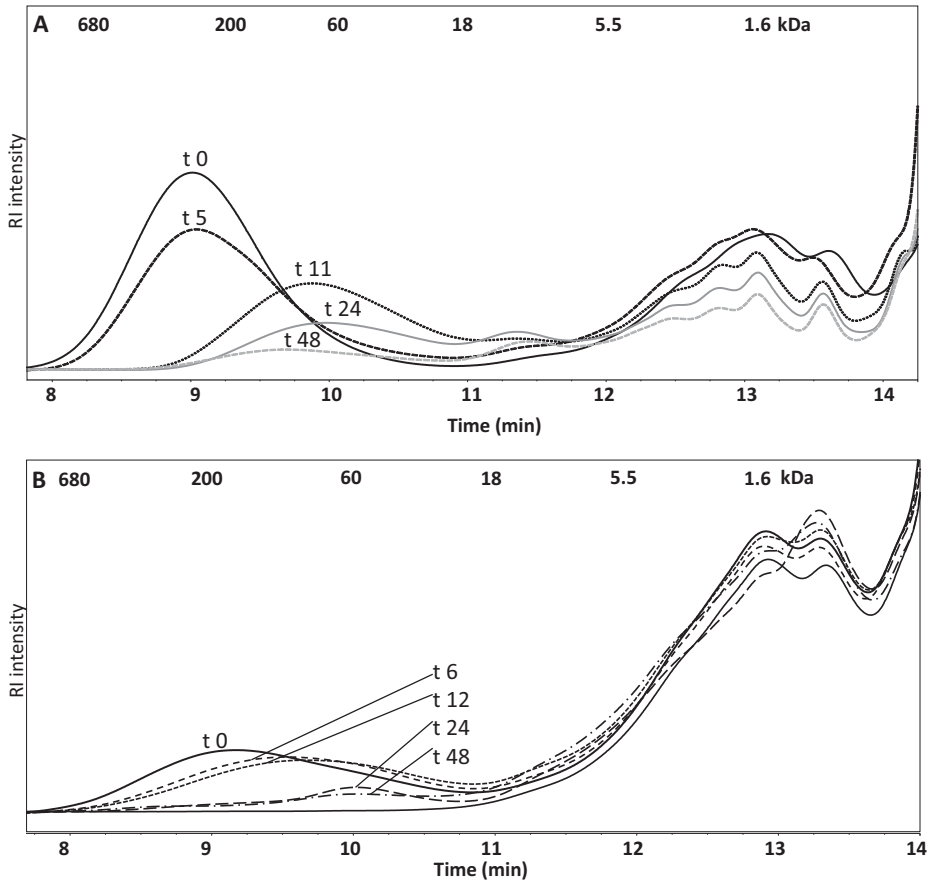

Figure 4. HPSEC PS profile of barley β-glucan (A) (0, 5, 11, 24, 48 h) and SBP (B) (0, 6, 12, 24, 48 h) fermentation digesta.

amounts (Fig. 5A). They were present in increasing amounts, because the fermentation of β-glucan was fast and determined by soluble and insoluble populations, leading to an imbalance in production and utilization of soluble intermediate products. The degradation products eluted mainly between retention time (R_t) 22 and 50 min, and could not be fully separated. These oligomers and disappear over fermentation time, which can be related to the fermentation of first all soluble, then followed by insoluble β-glucans at later stages of fermentation.

The fermentation digesta of 11 and 48 h consisted of similar soluble degradation products, but only the digesta of 11 h induced large amounts of cytokines. Also the 24 h fermentation digesta induced large amounts of cytokines, consisting of mainly soluble IMWs in comparison to the 11 h digesta. The only common molecules present in the 11 and 24 h digesta are the LMWs (eluting between R_t 25 and 35 min) and were in lower amounts present in the 48 h digesta and could thus be responsible for the immune stimulation described.

3.5.2 SBP fermentation

Contrary to the formation of degradation products during the β-glucan fermentation, almost no soluble OS were formed during the SBP fermentation (Fig. 5B). Only at 48 h of fermentation galacturonic acid was detected. Since the fermentation of SBP is rather slow, it could be the case that easily degradable OS metabolites were immediately utilized by the bacteria and thus not detected. The preferential fermentation of soluble fiber over insoluble fiber has been also observed for pectins present in chicory root pulp [17]. As only large soluble PS (HPSEC) were present at 48 h fermentation, the stimulation of cytokine secretion in BMDCs was due to large soluble PS molecules (ca. 60 kDa) (HPSEC) (Fig. 4).

Overall, barley β-glucan and especially SBP are rather slowly fermentable PSs under the current experimental conditions. For β-glucan this was probably due to the partial insolubility [17]. For SBP the explanation could be, besides its partly insolubility, that SBP is a rather structurally complex and difficult to degrade PSs. The potential benefit of slow fiber fermentation is that bioactive degradation products could be present in the intestine for a longer time to interact with epithelial and immune cells. In addition, fermentation is also shifted to the more distal colon parts [33].

3.6 Fermentation profile of SBP on monosaccharide constitution level

As it was observed that the molecular structure is also of importance for immunomodulatory activity and SBP consists of

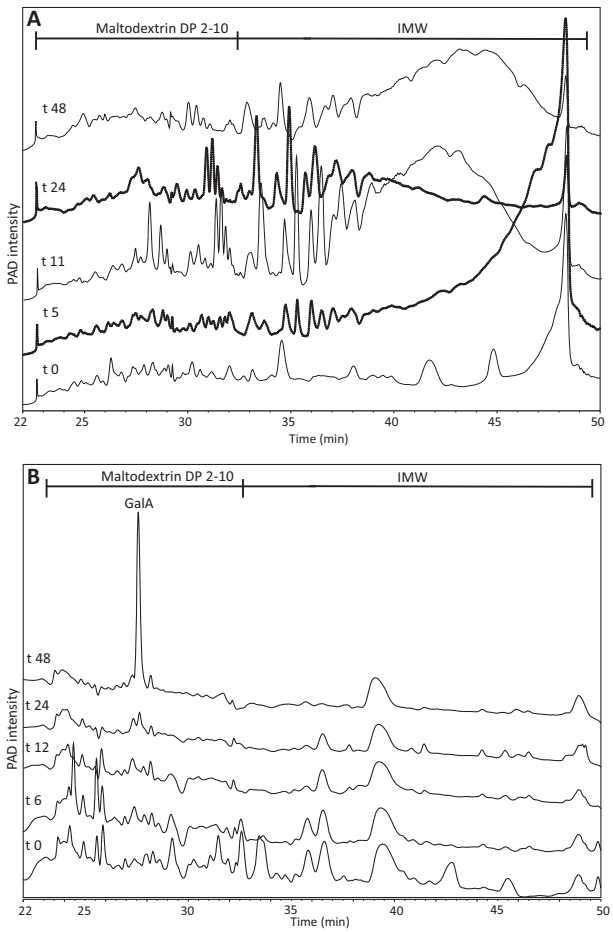

Figure 5. HPAEC elution profiles of barley β-glucan fermentation digesta (A) (0, 5, 11, 24, 48 h) and SBP fermentation digesta (B) (0, 6, 12, 24, 48 h).

many different monosaccharides, it was of interest to analyze which of the glycan moieties present in the oligo- and PSs, were fermented quickly and which were still present in the digesta and not utilized by the microbiota.

Figure 6 and Supporting Information Table 3 show the utilization of soluble and insoluble arabinose, galactose, and galacturonic acid, the main monosaccharides in SBP. The amounts of soluble galacturonic acid rich fragments decreased mostly only after 12 h of fermentation, while soluble polymers rich in galactose and arabinose decreased continuously up to 24 h. For the rest of the fermentation soluble galactose increased, which might originate from the degradation of the insoluble pectin fraction. Insoluble polymers rich in galacturonic acid, galactose, and arabinose increased during the first 12 h of fermentation and were subsequently almost completely utilized at 48 h.

The immune-stimulating SBP digesta of 48 h consisted of a high amount of soluble galactose rich fragments, which suggests that soluble pectin of equal ratios of galacturonic acid and galactan could be an immune-stimulating compound.

Furthermore, the constituent monosaccharide analysis of the SBP digesta showed that no rhamnose was present in the soluble digesta. This indicates that the RG I backbone is present only in the insoluble fraction, which is confirmed by the high amounts of arabinose and galactose, which are known to be attached to the RG I backbone [12] (Supporting Information Table 3).

3.7 Possible molecular characteristics of immune-active dietary fiber

In the highly immunomodulating β-glucan digesta, two soluble degradation populations were present (60 kDa and below DP 20). An increase in immunomodulation was also observed before for enzymatically degraded barley β-glucans having lower molecular masses (unpublished data). Amounts of insoluble glycans are unknown, but assumed to be present and also to be immunomodulatory.

In the highly immunomodulating SBP digesta, soluble galactan side chains and insoluble galacturonic acid backbones were present. The total amounts of SPB and its degradation products are negatively correlated with the amounts of induced cytokines. This also indicates that SBP degradation products are more immune modulating than the parental fibers. Furthermore, for SBP a trend between increased amounts of cytokines and an increase in insoluble fibers was observed.

The induced cytokine amounts are ca. 1.6- to 5.3-fold larger for barley β-glucan than SBP digesta, depending on the type of cytokine and fermentation time point. This shows that there are differences in immunomodulatory activity between the PS and their glycan degradation products, as well as between the two different fibers. This could be related to molecular differences. In addition to the immunomodulatory effect of the parental fibers, the difference in fiber fermentation may have implications for in vivo effects upon ingestion of these fibers, and could lead to differences in health effects.

4 Concluding remarks

In vitro fermentation digesta can be directly incubated with BMDCS from mice lacking both TLR2 and TLR4 receptors in order to study the immunostimulatory effect of dietary fibers and their microbiota-generated degradation products. This is one of the first studies investigating the immune response to complex fermentation digesta. Glycosidic soluble and insoluble fermentation metabolites were more immunomodulating than the parental fibers. This may have implication for health effects in human individuals when ingesting different dietary fibers. This also highlights the importance and need for innovative fermentation models integrating immune cells. Furthermore, detailed fermentation studies of dietary fibers are needed, including the characterization of degradation

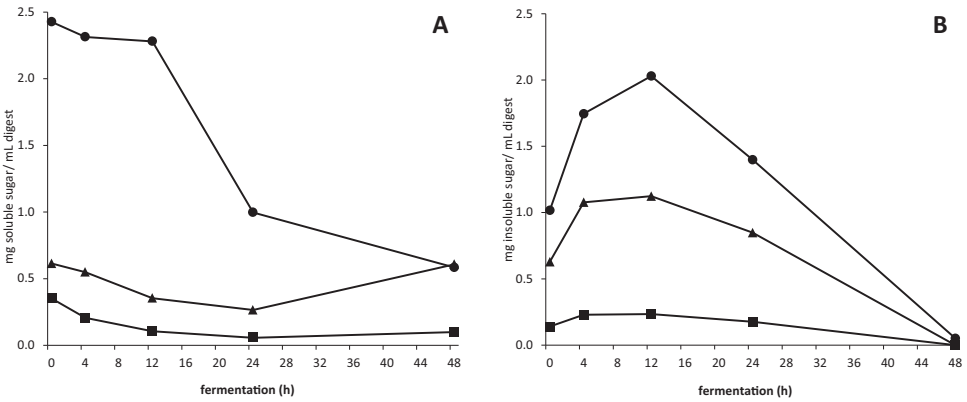

Figure 6. Amounts of soluble (A) and insoluble (B) arabinose (■), galactose (▲), and galacturonic acid (●) present as oligo- or PS in the SBP fermentation digesta.

products, in order to make conclusions about beneficial health effects of dietary fibers.

All authors participated in the design of the study. C.R. prepared samples for immunoassays, performed the fermentation, characterized the substrates, performed data analysis and interpretation, and wrote the manuscript. N.T. performed the immunoassays. K.V., H.S., J.W., and H.G. contributed to data interpretation and the manuscript.

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