Review

# Fecal water from ileostomic patients consuming oat β-glucan enhances immune responses in enterocytes

**Julian D. Ramakers[1], Julia J. Volman[1], Maria Biörklund[2], Gunilla Önning[2], Ronald P. Mensink[1] and Jogchum Plat[1]**

[1] Department of Human Biology, Nutrition and Toxicology Research Institute Maastricht (NUTRIM), Maastricht University, Maastricht, The Netherlands
[2] Biomedical Nutrition, Center for Chemistry and Chemical Engineering, Lund University, Lund, Sweden

Yeast, fungal, and dietary β-glucans have immune-modulating effects *in vitro* and *in vivo*, as thought, mainly by affecting leukocytes; however, effects of oat β-glucan on enterocytes have never been studied. As recognized, supplying oat β-glucans as such to cells in culture directly is difficult because of solubility problems. Therefore, six ileostomic patients consumed, in random order, a control diet or an oat β-glucan enriched diet (5 g) and from the collected ileostomic content, fecal water was prepared and added to two small intestinal cell lines (INT407, Caco-2) and two colon cell lines (HT29, T84) together with a cytokine cocktail (IL-1β + INFγ + TNFα). Several parameters reflecting immune-modulation were measured. As compared to placebo fecal water, β-glucan enriched fecal water significantly increased IL-8 production in HT29 (5.0%; $p = 0.046$) and INT407 cells (22.0%; $p = 0.028$). Intercellular adhesion molecule (ICAM)-1 expression increased in T84 (11.0%; $p = 0.028$) and Caco-2 cells (20.4%; $p = 0.075$). These immune-stimulating effects were confirmed by enhancement of inflammatory expression profiles, as determined with an antibody array. Our findings show immune enhancement by fecal water from ileostomic patients consuming oat β-glucan both in small intestinal and colon cell lines after stimulation, which is in agreement with documented effects in leukocytes. Whether these immune-stimulating effects on enterocytes contribute to the enhanced protection of the host against invading pathogens as observed both in animals and in humans, as well as the underlying mechanism, needs further evaluation.

**Keywords:** ICAM-1 / Ileostomy / Interleukin-8 / Intestinal epithelial cells / Oat β-glucan

Received: August 17, 2006; revised: October 10, 2006; accepted: October 12, 2006

## 1 Introduction

β-Glucans are carbohydrates consisting of linked glucose molecules with a molecular mass between 50 and 2300 kDa. They are major structural components of not only the cell walls of yeast and fungi, but also of some cereals such as barley and oat. In nature, there are many different forms of β-glucans varying in length, molecular mass, tertiary structure, and degree of branching. In this respect, the cell wall glucans of yeast and fungi consist of β-(1→3)-linked glucopyranosyl residues with small numbers of (1→6)-linked branches, whereas the oat endosperm cell walls contain unbranched β-glucans with β-(1→3) and β-(1→4) linkages [1–3]. These characteristics influence their effects on physiological functions such as lipid and glucose metabolism, and inflammatory processes.

It is now well established that fungal β-glucans initiate – by a so far not completely understood mechanism – a very potent immune response in leukocytes [2]. Less is known about the immune-modulating effects of β-glucans from oats and barley. Estrada *et al.* [1], however, have demonstrated that culturing macrophages in the presence of oat β-glucans enhanced the production of IL-1α in a dose- and time-dependent manner [1]. Also the production of IL-2, INF (INF)γ and IL-4 in cultured spleen cells was increased by oat β-glucan [1]. *In vivo* studies demonstrated that oat

**Correspondence:** Dr. Jogchum Plat, Department of Human Biology, Nutrition and Toxicology Research Institute Maastricht (NUTRIM), Maastricht University, P. O. Box 616, NL-6200 MD, Maastricht, The Netherlands
**E-mail:** j.plat@hb.unimaas.nl
**Fax:** 0031-433670976

**Abbreviations: CSF,** colony stimulating factor; **FCS,** fetal calf serum; **ICAM-1,** intercellular adhesion molecule-1; **MCP,** monocyte chemotactic protein; **MW,** molecular weight; **PS,** penicillin streptomycin; **TNFα,** tumor necrosis factor α

β-glucan enhanced resistance towards bacterial challenges and intestinal parasitic infections [1, 4–6]. All together, these findings indicate that β-glucans, not only from yeast and fungi but also from oat, have immune-stimulating effects *in vitro* and *in vivo*.

Moreover, Brown and Gordon [7], have recently suggested that high molecular weight (MW) and/or particulate β-glucans from fungi directly activate leukocytes, while low MW β-glucans from fungi only modulate the response of cells when they are stimulated with *e.g.* cytokines. Whether the effects of a low MW oat β-glucans are also indirect, *i.e.*, comparable to those of low MW fungi β-glucans, is unknown. In our hands, however, the fecal water with and without low MW oat β-glucans did not induce an immune-stimulation without the presence of an additional inflammatory trigger. Therefore, in this study we have chosen to evaluate immune-modulating effects of a low MW oat (1→3), (1→4) β-glucan (60 kDa) not as an inducing agent, but particularly as modifier of a cytokine-stimulated condition.

Most *in vitro* studies evaluated the effects of β-glucans in different leukocytes populations; however, effects on enterocytes have never been examined. Since it is inevitable that enterocytes play an important role in the intestinal defense against pathogens, the present study evaluated the immune-modulating effects of oat β-glucan on human enterocytes. It is however very difficult to study the effects of β-glucans in cell cultures directly, because of problems with solubility [8]. Therefore, to study the effects of oat β-glucan in a physiological matrix, ileostomic patients consumed, in random order, a control diet or an oat β-glucan enriched diet. From the collected illeostomic contents fecal water was prepared and added together with a cytokine cocktail (IL-1β, INFγ and tumor necrosis factor α (TNFα)) to enterocyte cell lines. We have deliberately chosen to study the effects in four different enterocyte cell lines (two small intestinal cell lines and two colon cell lines) to exclude the possibility that the presence or absence of the effects found were cell line specific. Next, the cells as well as the supernatants were analyzed for different inflammatory parameters. By this approach we were able to evaluate the effects of dietary oat β-glucan consumption under physiological conditions without making a distinction between direct (*i.e.*, β-glucan-enterocyte interactions) or indirect effects (*i.e.*, factors present in ileostomic content derived from effects of β-glucan in the intestinal tract of the patients). As far as we know, this is the first study showing immune-enhancing effects of oat β-glucans in enterocytes by such a physiological approach.

## 2 Materials and methods

### 2.1 Study design

Six patients (three males and three females) with an ileostomy participated in a double blind, placebo-controlled intervention trial with a crossover design (Fig. 1A). All subjects were proctocolectomized for ulcerative colitis. The median time since surgery was 6 years (range: 1–17 year). The mean age of the subjects was 51 years (range: 38–74 year). On the first day of the first period, all subjects received a standardized diet (three main meals and three snacks). On the second day, three subjects received again a standardized diet, but in addition, at breakfast and at lunch, a beverage (250 mL) enriched with β-glucan (2.5 g, *i.e.*, 5 g in total). The other three subjects received a placebo beverage enriched with rice starch. One week later, regimes were crossed over. The nutrient compositions of the two diets are presented for men and women in Table 1. During the second day of each period, ileostomic contents were collected 15 min before the standard meals at 7.45, 11.45, 15.45, 19.45, 21.45 and at 7.45 h the following morning. The ileostomic contents were sealed in a plastic jar immediately after each collection and put on dry ice. The next morning the ileostomic contents were weighed, freeze-dried, weighed again, homogenized, and stored at −20°C. Ethical approval of the study protocol was obtained from the local Research Ethics Committee of Lund University. All subjects gave their written informed consent before participating.

β-Glucan was isolated from Swedish oats (oats var. Sang from Cerealia (Sweden)) at Cereal Base Ceba AB (Lund, Sweden), as described [9]. Briefly, oat bran was crushed and milled with water and treated with amylases and proteases, while at the end insoluble fibers were removed by ultra-filtration. The remaining β-glucan fraction was freeze-dried, mixed with water, and used for the production of beverages with a β-glucan concentration of 1% w/w. The β-1→3)-(1→4)-linked unbranched soluble glucan had a mean MW of 60 kDa, determined by VTT Biotechnology, Technical Research Centre of Finland (Espoo, Finland), by SEC-HPLC using μHydrogel columns, NaOH-eluent, and postcolumn calcofluor staining with fluorescence detection, as described in ref. [10]. Control beverages were prepared using rice starch, while sucrose, glucose syrup, and rapeseed oil were added to balance the nutrient composition of the two experimental products.

### 2.2 Ileostomic contents and fecal water

The ileostomic contents from each subject obtained at each period were freeze-dried, after which the MWs and the concentrations of the β-glucan in the ileostomic contents were determined, as mentioned above.

The ileostomic contents from each subject, used for the cell stimulations, were pooled over the 24-h periods, thereby correcting for differences in volumes from each interval. Fecal water was prepared by dissolving the pooled freeze-dried ileostomic contents in PBS, which was subsequently rotated for 5 h. Next, the dissolved illeostomic contents were centrifuged for 11.5 min at 13 500 rpm, 17°C. The supernatant (further called fecal water) was carefully

 

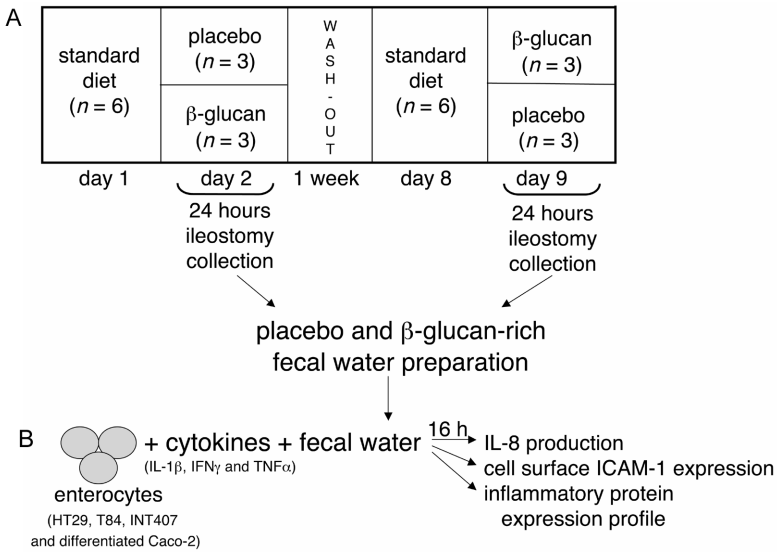


**Figure 1.** Study design. To study immune-modulating effects of oat β-glucan on enterocytes in a physiological matrix, (A) ileostomic patients consumed a diet enriched with β-glucan or placebo and ileostomic contents were collected. These ileostomic contents were used to prepare fecal water, which (B) was used in an *in vitro* study of stimulated enterocytes. For the first *in vivo* part (A), all ileostomic patients ($n = 6$) received a standardized diet (diet composition, see Table 1) on the first day of the first period. On the second day, three subjects received a standardized diet and at breakfast and at lunch a beverage (250 mL) enriched with β-glucan (2.5 g, *i.e.*, 5 g in total), while the other three subjects received a placebo beverage enriched with rice starch. One week later, regimes were crossed over. During this second day until the following morning (24 h in total) ileostomic contents were collected at fixed intervals. Fecal water was prepared by dissolving 24 h pooled freeze-dried ileostomic contents in PBS as described in the methods section and subsequently used for *in vitro* cell stimulation experiments. For this second *in vitro* part (B), placebo and β-glucan fecal water was added to four enterocyte cell lines (HT29, T84, INT407, and differentiated Caco-2) which were subsequently stimulated with a proinflammatory cytokine cocktail (IL-1β, INFγ and TNFα) for 16 h. After 16 h the culture medium was collected for measuring IL-8 production and inflammatory protein expression profiles and living cells were used for cell membrane intercellular adhesion molecule (ICAM)-1 protein expression measurements.

**Table 1.** Nutrient composition of the diets

| | Men ($n = 3$) | | Women ($n = 3$) | |
|---|---|---|---|---|
| | Placebo diet | β-Glucan diet | Placebo diet | β-Glucan diet |
| Energy (kcal) | 2170 | 2170 | 1813 | 1813 |
| Protein (g) | 98 | 98 | 89 | 89 |
| Protein (En%) | 18 | 18 | 20 | 20 |
| Fat (g) | 65 | 65 | 53 | 53 |
| Fat (En%) | 27 | 27 | 26 | 26 |
| Carbohydrates (g) | 300 | 300 | 241 | 241 |
| Carbohydrates (En%) | 55 | 55 | 53 | 53 |
| Fibers (g) | 11 | 16 | 8 | 13 |
| β-Glucan (g) | 0 | 5 | 0 | 5 |

collected and used for the *in vitro* cell stimulation experiments. The pH of each fecal water sample was determined.

### 2.3 Intestinal cell cultures

Four different human-derived intestinal cell lines – two small intestinal and two colon cell lines – were used. The human small intestinal cell line INT407 was obtained from the European Tissue Type Collection (ETTC) and the human cell line Caco-2 from the American Tissue Type Collection (ATTC). INT407 cells were cultured in minimum essential medium (MEM) (Invitrogen Corporation, Paisley, UK) supplemented with 10% heat inactivated fetal calf serum (FCS) (Greiner Bio-one, Frickenhausen, Germany) and 1% penicillin streptomycin (PS) (Invitrogen Corporation). Caco-2 cells were cultured in DMEM (Invitrogen Corporation) supplemented with 10% FCS, 1% PS, 1% sodium pyruvate (SP) (Invitrogen Corporation), and 1%

 

nonessential amino acids (NEAA) (Invitrogen Corporation). After 18 days culturing of Caco-2 cells in normal DMEM, they were differentiated into small intestinal enterocytes [11]. The human colon cell lines HT29 and T84 were kindly supplied by Professor Dr. W. A. Buurman (Department General Surgery, Maastricht University, The Netherlands). HT29 cells were cultured in RPMI 1640 medium (Invitrogen Corporation) supplemented with 10% FCS and 1% PS. T84 cells were cultured in DMEM nut mix F-12 (DMEM/F12) (Invitrogen Corporation) supplemented with 10% FCS and 1% PS. All cells were cultured at 37°C in a 5% $CO_2$ humidified atmosphere. Cells were refreshed every second day and were separated by trypsine-0.03% EDTA (Greiner Bio-one) when they had reached 70–90% confluency.

### 2.4 Cell cytotoxicity

Cell cytotoxicity was measured to determine the maximum concentration of fecal water that could be added to the cell lines without causing cytotoxic effects. For this, different concentrations of fecal water were prepared and tested in two assays, *i.e.*, (i) erythrocytes lytic activity and (ii) Phenol Red leakage across confluent Caco-2 cell monolayers.

#### 2.4.1 Erythrocytes lytic activity assay

Cytotoxic activity of fecal water was determined by evaluating hemolysis of erythrocytes. For this, human blood with heparin as anticoagulant – from the same volunteer for all experiments – was centrifuged for 10 min at 3500 rpm to obtain erythrocytes. Plasma was removed and the remaining erythrocytes were washed three times with PBS and centrifuged again for 10 min at 3500 rpm. The cytotoxicity of fecal water was determined by adding different concentrations of fecal water, prepared by dissolving different amounts of the ileostomic content, to the washed human erythrocytes suspension. After incubation for 45 min at 37°C, the tubes were centrifuged for 10 min at 3500 rpm. The supernatant was transferred to a 96-wells plate and diluted 20 times. The hemoglobin concentration in the supernatant, released after lysis of the erythrocytes, was measured spectrophotometrically at 540 nm. Percentage hemolysis was calculated compared to a calibration curve, made from erythrocytes suspension incubated with ascending concentrations of distilled water in PBS (0–100% hemolysis). The concentration at which 2% lysis occurred was defined as the critical lytic concentration (CLC) [12]. Concentrations below the CLC were determined as noncytotoxic and were used for further experiments.

#### 2.4.2 Phenol Red leakage across confluent Caco-2 monolayers

Cytotoxicity of fecal water was also measured by Phenol Red leakage across confluent Caco-2 monolayers, which can be used as a good marker for cell confluence and tight junction formation and is an easy-to-use alternative for the electrical measurement of the transepithelial electrical conductance [13]. For this, Caco-2 cells were cultured in a polarized transwell system (Costar, Badhoevedorp, The Netherlands). The upper apical chamber contained DMEM (Invitrogen Corporation) with Phenol Red and the lower basolateral compartment DMEM without Phenol Red. Disturbance of the confluent Caco-2 cell monolayer due to cytotoxicity of the fecal water resulted in a flux of the small Phenol Red molecule (MW = 354) across the epithelium, which could be measured in the basolateral compartment by spectrophotometry at 479 nm [13]. When the cells were confluent as indicated by the absence of Phenol Red leakage, cells were refreshed with medium containing fecal water at different concentrations. DMSO (10%) and PBS were used as positive and negative controls, respectively. Cells were incubated for 6 h with fecal water, 10% DMSO or PBS added apically. Next, the basolateral medium was collected, and refreshed after which the cells were incubated for another 18 h with the same compounds. The Phenol Red concentration in the basolateral medium (at 6 and 18 h) was measured. Values were corrected for Phenol Red free medium and compared to the 10% DMSO and PBS controls. Concentrations without Phenol Red leakage were determined as noncytotoxic and were used for further experiments.

### 2.5 Cell stimulations

To evaluate the immune-modulating effects of fecal water with β-glucan, *in vitro* cell stimulation experiments were conducted as represented schematically in Fig. 1B. For this HT29 and T84 cells were plated in six-well plates at an initial density of $1.0 \times 10^6$ cells/mL, INT407 at $0.6 \times 10^6$ cells/mL, and Caco-2 at $0.5 \times 10^6$ cells/mL in a total volume of 1.5 mL. When the HT29, T84, and INT407 cells had reached 70–90% confluency and the Caco-2 cells had differentiated for 18 days, the culture medium was replaced by medium containing fecal water (with or without β-glucan) and a cocktail consisting of the proinflammatory cytokines INFγ (100 U/mL), IL-1β (50 U/mL), and TNFα (10 ng/mL). After 16 h of incubation, culture medium was collected for analysis of IL-8 concentrations and inflammatory protein expression profiles, while the living cells were used to determine cell surface intercellular adhesion molecule (ICAM)-1 protein expression.

### 2.6 IL-8 ELISA and inflammatory protein expression profiles

IL-8 concentrations in culture supernatants were determined using an ELISA as described in [14]. Briefly, plates (Greiner Bio-one) were coated with monoclonal murine antihuman IL-8 antibodies. Recombinant human IL-8 was used for standard curves. Immobilized IL-8 was detected

 

using a specific biotinylated rabbit antihuman IL-8 polyclonal antibody, followed by the addition of peroxidase-conjugated streptavidin (Zymed Laboratories, San Francisco, Canada) and tetramethylbenzidine (TMB) substrate (Kirkegaard & Perry Laboratories, Gaithersburg, USA). The detection limit of the ELISA was 5 pg/mL.

Cell culture supernatants were also used to evaluate expression profiles of multiple inflammatory proteins using the human cytokine antibody array III (Ray Biotech Inc., Norcross, USA). First, cell culture media of all six subjects after 16 h incubation with fecal water containing β-glucan or placebo were pooled. Thus, eight arrays (HT29, T84, INT407, Caco-2 with β-glucan or placebo) were analyzed. One milliliter of the pooled samples was added to the array membranes. After incubating and washing, the cytokine-bound membrane was incubated with a cocktail of biotin-labeled antibodies, followed by the addition of horseradish peroxidase-conjugated streptavidin. Array spot intensity was detected by using a LAS-3000 Lite Image reader (Raytest GmbH, Straubenhart, Germany) based on chemiluminescene imaging. Intensity of the spots was quantified in arbitrary units by densitometry using Aida software version 3.50 (Raytest GmbH), thereby correcting for background staining of the gel. Comparison of protein expression profiles was possible after normalization of each spot on an array using the positive controls, provided by the manufacturer. The cytokines used for stimulation (INFγ, IL-1β and TNFα) were excluded from the analysis.

Using the same protocol, this array was also used to determine the amounts of inflammatory proteins possibly present in fecal water. For this, fecal water containing β-glucan or placebo was prepared for each subject and pooled *per* dietary period. For analysis, the fecal water was not diluted.

### 2.7 Flow cytometry analysis of intercellular adhesion molecule-1 (ICAM-1)

ICAM-1 protein expression on the cell surface of living cells was measured by flow cytometry. After 16 h incubation, cells were washed three times with PBS and detached with trypsine-0.03% EDTA. When the cells were detached, the medium was added and cell suspensions were centrifuged for 5 min at 1200 rpm at room temperature, followed by resuspending the pellets in 500 μL of PBS-1% BSA. Cells were counted and diluted to $1 \times 10^6$ cells/mL in PBS-1% BSA. Recombinant-phycoerythrin (R-PE)-conjugated mouse-anti-human CD-54 mAb (anti-ICAM-1) or isotype-matched control antibody (Becton Dickinson Biosciences, San Diego, USA) 20 μL/$10^6$ cells was added and incubated for 30 min on ice in the dark. Next, cell suspensions were centrifuged for 5 min at 1500 rpm and pellets were resuspended in 500 μL PBS-1% BSA. Because almost all cells were ICAM-1 positive, although they greatly differed in the amount of ICAM-1 expression, the mean fluorescence of 10 000 living cells was measured and analyzed with the FACSort (Becton Dickinson, Franklin Lakes, USA) and CellQuest analysis software. Percentage living cells were not different for the different samples and the isotype control antibody showed fluorescence below the threshold.

### 2.8 Statistical analysis

Effects on IL-8 production and ICAM-1 expression were examined with the nonparametric, Wilcoxon signed ranks test. For data presentation, effects from the β-glucan enriched fecal water were also expressed as percentages relative to those of the placebo fecal water. All statistical analyses were performed using SPSS 11.0 (SPSS, Chicago, IL, USA). Values of $p < 0.05$ were considered statistically significant.

## 3 Results

### 3.1 Characteristics of ileostomic content and fecal water

The mean MW of the β-glucan in the beverages and in the ileostomic content of four subjects was 60 kDa. For two subjects, only a very slight decrease in MW was observed, indicating that, overall, the β-glucan was not degraded while passing the small intestine.

Fecal water concentrations that were not cytotoxic, as determined by erythrocyte lysis, were in agreement with Phenol Red leakage assays (data not shown). Experiments in all cell lines were, therefore, carried out with a concentration of 1.44 mg/mL redissolved, freeze-dried ileostomic content in PBS. The calculated concentrations of β-glucan added to the cells varied from subject to subject between 0.12 and 0.18 mg/mL.

The mean pH of fecal water containing β-glucan was not significantly different as compared to placebo fecal water (7.4 ± 0.2 and 7.3 ± 0.2 (mean ± SD), respectively).

To exclude the possibility that the β-glucan enriched fecal water already contained proinflammatory proteins, which may have confounded the results, we measured inflammatory proteins with the antibody array. However, no detectable amounts of inflammatory proteins were present in the fecal water from the β-glucan or placebo period (data not shown).

### 3.2 IL-8 production

Addition of the β-glucan enriched fecal water, without stimulation with cytokines, did not induce IL-8 production in any of the four intestinal cell lines (data not shown). However, when β-glucan enriched fecal water was added in combination with a cytokine mixture, we found enhancing effects as compared to placebo fecal water, illustrating that our low MW oat (1→3), (1→4) β-glucan (60 kDa), indeed,

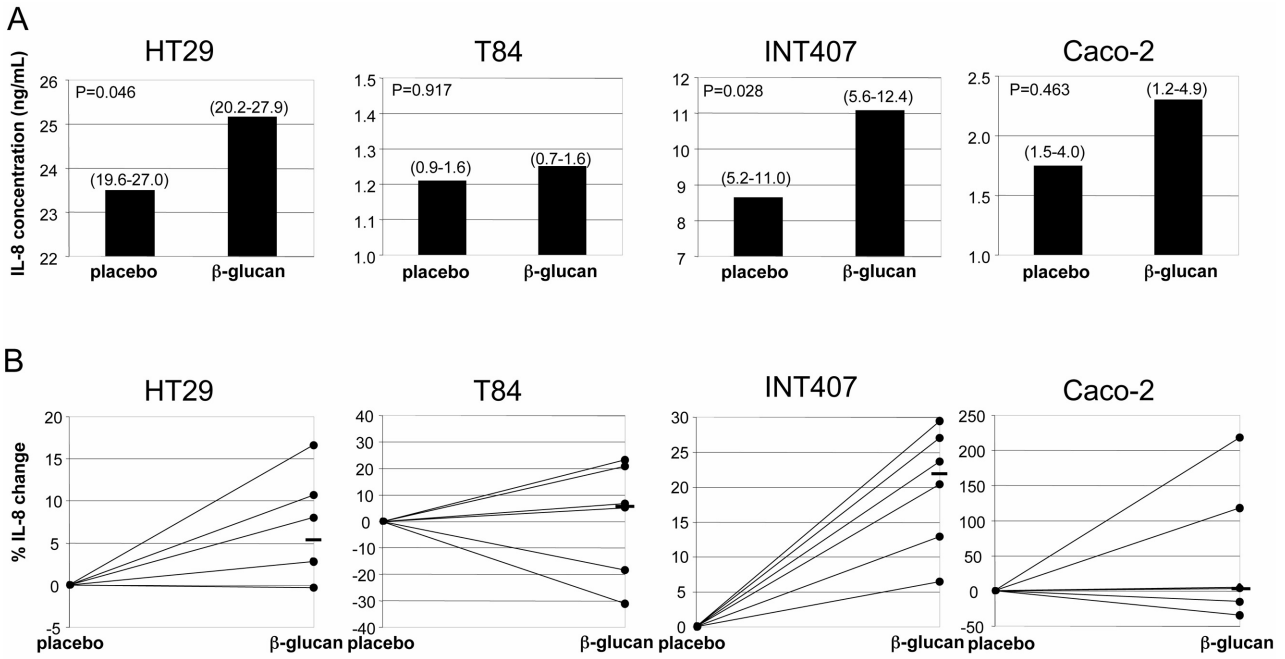


**Figure 2.** β-Glucan containing fecal water increased IL-8 production by enterocytes. IL-8 production by HT29, T84, INT407, and Caco-2 cells after 16 h incubation with fecal water containing β-glucan or placebo and stimulation with cytokines (A, B). (A) The median (and ranges) of IL-8 concentrations (ng/mL) after β-glucan and placebo fecal water and (B) percent changes in IL-8 concentration of each individual after fecal water containing β-glucan corrected for placebo are shown for the four enterocyte cell lines. In panel B, each subject is represented by a dot and the median is represented by a line.

is not an inducing agent but a modifier of a cytokine-stimulated condition. Figure 2A shows the median IL-8 concentrations (and ranges) after placebo and β-glucan fecal water for all subjects together in the four cell lines, while in Fig. 2B, percent changes for the β-glucan fecal water for each subject individually corrected for placebo fecal water are shown. β-Glucan containing fecal water from five subjects increased IL-8 production by HT29 cells. For one subject, no change was found. Overall, this resulted in a significant median increase in IL-8 production of 5.4% (range: −0.3%, 16.6%; $p = 0.046$). Comparable to HT29 cells, IL-8 production by INT407 cells was increased for all six subjects after addition of fecal water containing β-glucan, resulting in a significant increase of 22.0% (range: 6.5%, 29.4%; $p = 0.028$). In contrast, IL-8 production by T84 cells and Caco-2 cells was not significantly changed (median: 5.9%; range: −31.1%, 23.1%; $p = 0.917$ and median: 4.2%; range: −34.8%, 218.3%; $p = 0.463$, respectively).

### 3.3 Cell surface ICAM-1 protein expression

In line with effects on IL-8 production, addition of the β-glucan enriched fecal water without stimulation with cytokines did not induce ICAM-1 expression in any of the four intestinal cell lines (data not shown), while stimulating effects on ICAM-1 expression were visible in the presence of cytokine stimulation. Figure 3A shows the ICAM-1 expression after placebo and β-glucan fecal water for all subjects together in each of the four cell lines and in Fig. 3B percent changes for the β-glucan fecal water for each subject individually corrected for placebo fecal water are shown. These figures show that the β-glucan containing fecal water from all subjects increased ICAM-1 expression in T84 cells. This resulted in a significant increase of ICAM-1 expression for T84 (median: 11.0%; range: 3.8%, 31.1%; $p = 0.028$). ICAM-1 expression in Caco-2 cells and INT407 cells was increased for five out of the six subjects, with a median ICAM-1 increase of 20.4% (range: −8.8%, 40.5%; $p = 0.075$) and 16.7% (range: −19.0%, 30.0%; $p = 0.249$), respectively. ICAM-1 expression in HT29 cells, however, was not significantly changed (median: −0.4%; range: −8.4%, 10.8%; $p = 0.917$).

### 3.4 Inflammatory protein expression profiles

In general, β-glucan enriched fecal water increased the expression of almost all detectable cytokines, chemokines, colony stimulating factors (CSF) and growth factors compared to placebo fecal water (Fig. 4). No bar means that the protein was not detectable in the culture medium of that particular cell type or that there was no change in protein expression after β-glucan compared to placebo fecal water. When examining the expression profiles into more detail, it can be seen that especially the changes in chemokines

 

expressions were quite consistent. For example, in all cell lines expressions of all detectable monocyte chemotactic proteins (MCPs) were increased (MCP-2 and 3 expression not measurable in HT-29 cells and no MCPs measurable in Caco-2 cells), while that of MIG (monokine induced by INFγ), a chemokine that attracts T-lymphocytes, was decreased (not measurable in T84 and INT407 cells). The increases in IL-8 expressions agreed very well with those observed with the ELISA technique, confirming the validity of the antibody array. It should be noted, however, that

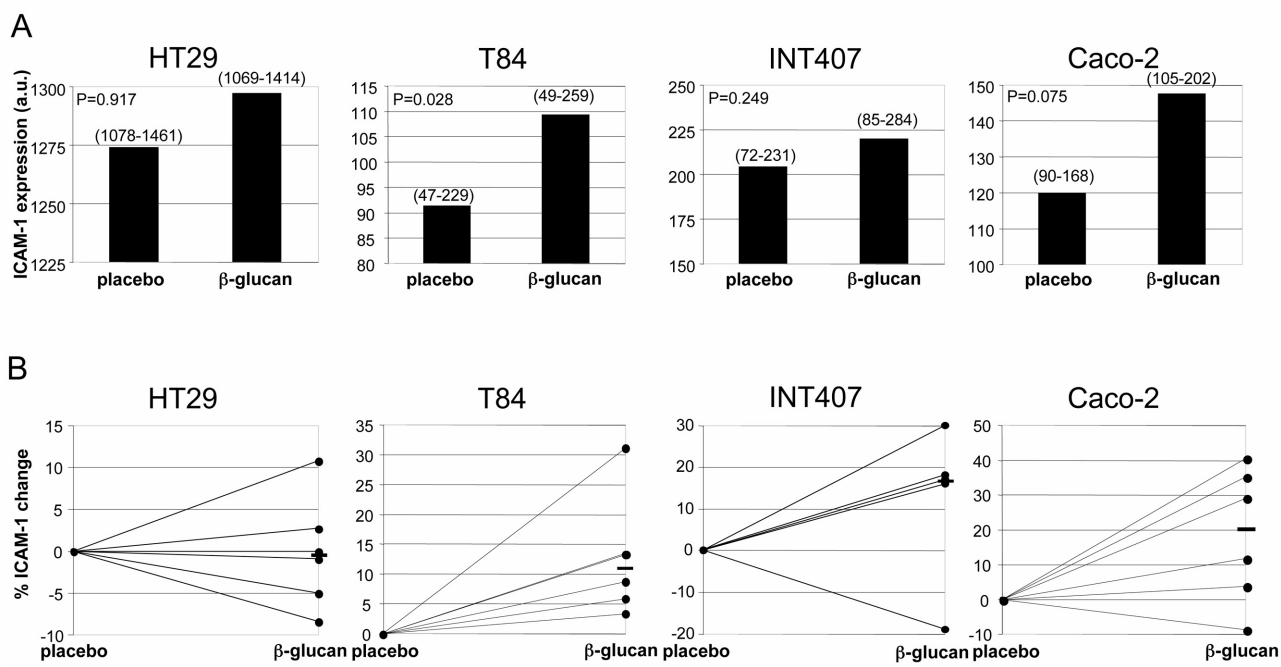


**Figure 3.** β-Glucan containing fecal water increased ICAM-1 expression in enterocytes. ICAM-1 expression in HT29, T84, INT407, and Caco-2 cells after 16 h incubation with fecal water containing β-glucan or placebo and stimulation with cytokines (A, B). (A) The median (and ranges) of ICAM-1 expression (arbitrary units) after β-glucan and placebo fecal water and (B) percent changes in ICAM-1 expression of each individual after fecal water containing β-glucan corrected for placebo. In panel B, each subject is represented by a dot and the median is represented by a line.

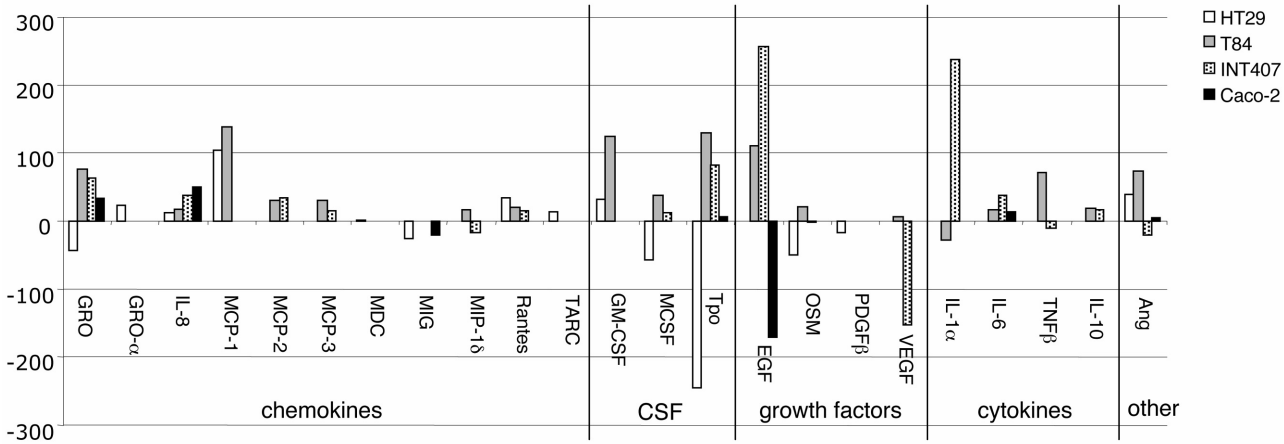


**Figure 4.** β-Glucan containing fecal water mostly increased the expression of various inflammatory proteins of enterocytes. Percent change in expression of chemokines, CSF, growth factors, and cytokines after β-glucan, as compared to placebo fecal water in HT29, T84, INT407, and Caco-2 cells as measured by an antibody array. For experimental details see Section 2. GRO, growth regulated protein; MDC, macrophage-derived chemokine; MIG, monokine induced by INFγ; MIP, macrophage inflammatory protein; RANTES, regulated upon activation normal T-cell expressed and secreted; TARC, thymus and activation-regulated chemokine; GM-CSF, granulocyte-macrophage CSF; MCSF, macrophage CSF; Tpo, thrombopoietin; EGF, epidermal growth factor; OSM, oncostatin M; PDGF, platelet-derived growth factor; VEGF, vascular endothelial growth factor; TNF, tumor necrosis factor; Ang, angiogenin.

the relative change measured with the antibody array was higher in Caco-2. This can be explained by the fact that for the antibody array pooled samples were used and the results therefore represent a mean value. For the ELISA, median values were presented. Changes in CSF and growth factor expressions were less consistent. The T84 cells showed an increased expression for all CSF and growth factors, whereas the HT29 cells showed a decreased expression for all these proteins except for GM-CSF. Also for INT407 and Caco-2 cells, increases and decreases for these proteins were observed.

## 4 Discussion

Not only β-glucans from the cell wall of fungi and yeast, but also β-glucans from oats have been shown to be potent immune stimulators, at least in macrophages and spleen cells [1]. However, effects on enterocytes have never been examined. Our results now show that fecal water prepared from ileostomic contents of patients consuming diets enriched with oat β-glucans, induced an immune-stimulating response in enterocytes both from small intestinal as well as from colonic origin. The finding that not all four cells lines responded significantly regarding all parameters, but the fact that nearly all responses observed in the different cell lines were a stimulation, strengthens our belief that oat β-glucans indeed enhanced immune responses in enterocytes. Since our primary goal was to see whether enterocytes responded to β-glucan enriched fecal water at all, we did not attempt to define the mechanism underlying this response in this phase. Therefore, as far as we know now, these findings could be ascribed to direct as well as indirect effects of oat β-glucan.

We measured immune-modulating effects of β-glucan by examining IL-8 production and ICAM-1 expression of enterocytes. IL-8 is a chemokine and ICAM-1 is an adhesion molecule whose expression is both increased in the case of an infection by pathogens. IL-8 production by enterocytes will induce recruitment of inflammatory cells such as neutrophils, basophils, and lymphocytes to the inflamed tissue [15]. ICAM-1 expression on enterocytes may help to keep leukocytes that have transmigrated through the epithelial layer towards the intestinal lumen in close contact with the intestinal epithelium [16]. This will prevent further invasion of the mucosa by pathogens. Fecal water with β-glucan increased the production of IL-8 and the cell surface expression of ICAM-1, which thus indicates an enhanced immune response. This agrees with the results of an *in vivo* study with C57BL/6 mice showing that oral administration of (1→3), (1→6)-β-glucan isolated from baker's yeast increased the number of intraepithelial lymphocytes in the intestine [17]. Although effects of yeast glucans and oat glucans may, because of structural differences, not as such be extrapolated to each other, the similarity in responses is suggestive.

Inflammatory signatures obtained from the antibody arrays suggest a potential role of other inflammatory players, because β-glucan elevated the expression of numerous inflammatory proteins. An important group of proteins that showed highly consistent elevations in all the four enterocyte cell lines were the MCPs. The elevation of these chemokines, which preferably attract monocytes, suggests a role of these cells (besides the granulocytes and lymphocytes attracted by IL-8) in the β-glucan induced an improvement in immune function as well. Our results therefore suggest that the elevated IL-8 production and increased ICAM-1 expression and, probably, also the increased production of MCPs by enterocytes may contribute to the increased resistance to pathogens after β-glucan consumption, as shown in mice [1, 4] and humans [18, 19]. No correlation between the β-glucan concentration in the fecal water and the overall inflammatory response (in all cell lines) of each subject was present, probably due to the small β-glucan concentration interval (0.12–0.18 mg/mL) and the small number of subjects. We showed that the four cell lines did not always respond in the same manner. For example, in T84 cells the IL-8 production was not significantly different, whereas ICAM-1 expression significantly increased. This suggests that individual inflammation markers are differently regulated in the various intestinal cell lines. Therefore, we decided to measure a whole range of inflammation-related markers in all four cell lines. Although there are differences between the cell lines and not all effects were significant, the overall conclusion is that they are nearly all in the same direction, indicating immune-stimulating effects of β-glucan enriched fecal water in enterocytes.

To what extent our findings can be extrapolated to other types of β-glucans is not known. β-Glucans are a complex group of molecules with different degrees of branching, polymer lengths, tertiary structures, and solubilities [3, 20]. We have used a soluble unbranched β-glucan with β-(1→3) and β-(1→4) linkages, that was extracted from oat with a mean MW of 60 kDa. Brown and Gordon have recently suggested that high MW and/or particulate β-glucans of fungi directly activate cells. In contrast, low MW β-glucans might not directly activate cells, but may modulate the response to another challenge. Results of these low MW β-glucans, however, are conflicting, as both primed and suppressed secondary responses have been reported [7]. There is no clear explanation for these different results, but it is known that small differences between β-glucan molecules besides MW also have an impact on their immune-modulating effects [7, 20]. Unfortunately, characteristics of the β-glucans used in other studies have not always been reported [7]. In the present study, we have now shown that a low MW oat (1→3), (1→4) β-glucan (60 kDa) in the physiological matrix of fecal water induced an enhanced immune response in enterocytes after cytokine stimulation. Moreover, the finding that addition of the β-glucan enriched

 

fecal water without stimulation with cytokines did not induce an inflammatory response (data not shown) supports the finding that low MW β-glucans can modulate, but not evoke, an inflammatory response. However, despite numerous barriers and mechanisms counteracting bacterial translocation and too strong inflammatory responses, it is very likely that there is always some translocation also in a normal situation [21]. Therefore, it is very likely that a local inflammatory micromilieu is always present in the intestine [21], suggesting that adding cytokines to stimulate the enterocytes might be considered rather physiological. With respect to the physiological relevance of the β-glucan induced immune enhancement, the finding that numerous immune mediators all change in the same direction, suggest that this can have a strong physiologic effect. However, in contrast to the *in vivo* situation in the intestines, the *in vitro* cultured enterocytes do not produce mucus, however mucus is not expected to prevent direct contact between enterocytes and β-glucan since β-glucan is water soluble and is expected to easily pass the mucus layer.

Dectin-1 plays a central role to explain the immune-modulating effects of β-glucans [3]. Of the four epithelial intestinal cell lines we have used, dectin-1 mRNA expression has been shown in Caco-2 cells [22]. As far as we know, the expression of dectin-1 in the other cell lines is not examined yet. The expression of dectin-1 on intestinal epithelial cells *in vivo* is contradictory. Presence and absence on mRNA and protein level and on murine and human intestinal cells have been reported [23–25]. Because it is not evident if dectin-1 is expressed on enterocytes, we cannot exclude the possibility that the observed effects of β-glucan are not mediated by dectin-1. The present study was not initiated to mechanistically examine the effects of β-glucan. Whether our results are dectin-1 mediated or due to indirect changes in the fecal water composition induced by oat β-glucan will hopefully be addressed in future studies.

As suggested above, the observed effects in our *in vitro* model may also be caused by an increased or decreased production of another, yet unknown, factor. If true, this implies that effects of β-glucan fecal water on enterocytes are indirect and would in that case never have been found when – if possible – the oat β-glucans were applied to the cells directly. As an example of indirect effects, β-glucan could have induced changes in inflammatory proteins in the fecal water. However, we could not identify any inflammatory proteins in the fecal water with the highly sensitive antibody array. Although the amount of LPS in the fecal water is not determined, effects of LPS as explanation for the observed changes in the intestinal cell lines used can be neglected since all cell lines were found nonresponsive to even high concentrations of LPS (data not shown). However, measuring LPS is not the solution since potential effects of other microbial products in fecal water cannot be ruled out. However, since each volunteer was his own control, potential disturbing effects of these factors are not very likely. Another possible explanation for the observed effects is an increased production of antimicrobial peptides like defensins, after consumption of the β-glucan. These peptides, which will then be present in elevated amounts in fecal water, may also influence intestinal inflammation by stimulating chemokine secretion and attracting leukocytes [26]. Another possible mechanism explaining the observed effects is the degradation of β-glucan into SCFA. SCFA, for example butyrate, are known to modulate the intestinal flora and intestinal inflammation [27]. However, in our study we worked with ileostomic patients and β-glucan did, therefore, not pass the colon, the only place where fibers can be degraded into SCFA by bacteria. Also the MW of β-glucan in the ileostomic content was not substantially changed, which excludes the formation of degradation products. It is therefore not likely that the reported effects are caused by differences in SCFA composition of the fecal water. Finally, it is known that consumption of β-glucan enriched diets increases fecal bile acid excretion [28]. If anything, however, bile acids will have immune-suppressive effects [29].

In conclusion, we found that β-glucan enriched fecal water enhanced the immune response in stimulated epithelial intestinal cell lines, which should allow the host to defend itself better against invading pathogens. This is to our opinion the first time that responsiveness of enterocytes towards β-glucans has been shown. Whether the reported effects of β-glucan enriched fecal water are direct effects of β-glucan (potentially *via* dectin-1) or indirect (*via* so far unknown mechanisms) need further investigation. Finally, extrapolation of our findings to the *in vivo* situation remains to be established.

*We thank Tapani Suortti and Marjatta Salmenkallio-Marttila (VTT Biotechnology, Technical Research Centre of Finland, Espoo, Finland) for analysis of the molecular weight of the β-glucans and Angeliki Öste-Triantafyllou (Cereal Base Ceba AB, Lund, Sweden) for helping with the formulation of the study products. We thank Dr. Erik Hertervig, Lund University Hospital, for recruiting the subjects. This work was supported by a grant from the Commission of the European Communities, project QLKI-CY-2000-00535.*

## 5 References

[1] Estrada, A., Yun, C. H., Van Kessel, A., Li, B. *et al.*, Immunomodulatory activities of oat beta-glucan in vitro and in vivo, *Microbiol. Immunol.* 1997, *41*, 991 – 998.

[2] Brown, G. D., Gordon, S., Fungal beta-glucans and mammalian immunity, *Immunity* 2003, *19*, 311 – 315.

[3] Brown, G. D., Herre, J., Williams, D. L., Willment, J. A. *et al.*, Dectin-1 mediates the biological effects of beta-glucans, *J. Exp. Med.* 2003, *197*, 1119 – 1124.

[4] Yun, C. H., Estrada, A., Van Kessel, A., Park, B. C. *et al.*, Beta-glucan, extracted from oat, enhances disease resistance against bacterial and parasitic infections, *FEMS Immunol. Med. Microbiol.* 2003, *35*, 67 – 75.

[5] Yun, C. H., Estrada, A., Van Kessel, A., Gajadhar, A. A. *et al.*, Beta-(1→3, 1→4) oat glucan enhances resistance to Eimeria vermiformis infection in immunosuppressed mice, *Int. J. Parasitol.* 1997, *27*, 329 – 337.

[6] Yun, C. H., Estrada, A., Van Kessel, A., Gajadhar, A. *et al.*, Immunomodulatory effects of oat beta-glucan administered intragastrically or parenterally on mice infected with Eimeria vermiformis, *Microbiol. Immunol.* 1998, *42*, 457 – 465.

[7] Brown, G. D., Gordon, S., Immune recognition of fungal beta-glucans, *Cell. Microbiol.* 2005, *7*, 471 – 479.

[8] Muller, A., Rice, P. J., Ensley, H. E., Coogan, P. S. *et al.*, Receptor binding and internalization of a water-soluble (1→3)-beta-D-glucan biologic response modifier in two monocyte/macrophage cell lines, *J. Immunol.* 1996, *156*, 3418 – 3425.

[9] Oste-Triantafyllou, A., Patent 6592914, Sweden, Method for the isolation of a beta-glucan composition from oats and products made therefrom, 2000.

[10] Suortti, T., Size-exclusion chromatographic determination of beta-glucan with postcolumn reaction detection, *J. Chromatogr.* 1993, *632*, 105 – 110.

[11] Hidalgo, I. J., Raub, T. J., Borchardt, R. T., Characterization of the human colon carcinoma cell line (Caco-2) as a model system for intestinal epithelial permeability, *Gastroenterology* 1989, *96*, 736 – 749.

[12] Lapre, J. A., Termont, D. S., Groen, A. K., Van der Meer, R., Lytic effects of mixed micelles of fatty acids and bile acids, *Am. J. Physiol.* 1992, *263*, G333 – G337.

[13] Jovov, B., Wills, N. K., Lewis, S. A., A spectroscopic method for assessing confluence of epithelial cell cultures, *Am. J. Physiol.* 1991, *261*, C1196 – C1203.

[14] Bouma, M. G., Stad, R. K., van den Wildenberg, F. A., Buurman, W. A., Differential regulatory effects of adenosine on cytokine release by activated human monocytes, *J. Immunol.* 1994, *153*, 4159 – 4168.

[15] Eckmann, L., Jung, H. C., Schurer-Maly, C., Panja, A. *et al.*, Differential cytokine expression by human intestinal epithelial cell lines: Regulated expression of interleukin 8, *Gastroenterology* 1993, *105*, 1689 – 1697.

[16] Huang, G. T., Eckmann, L., Savidge, T. C., Kagnoff, M. F., Infection of human intestinal epithelial cells with invasive bacteria upregulates apical intercellular adhesion molecule-1 (ICAM)-1) expression and neutrophil adhesion, *J. Clin. Invest.* 1996, *98*, 572 – 583.

[17] Tsukada, C., Yokoyama, H., Miyaji, C., Ishimoto, Y. *et al.*, Immunopotentiation of intraepithelial lymphocytes in the intestine by oral administrations of beta-glucan, *Cell. Immunol.* 2003, *221*, 1 – 5.

[18] Babineau, T. J., Hackford, A., Kenler, A., Bistrian, B. *et al.*, A phase II multicenter, double-blind, randomized, placebo-controlled study of three dosages of an immunomodulator (PGG-glucan) in high-risk surgical patients, *Arch. Surg.* 1994, *129*, 1204 – 1210.

[19] Babineau, T. J., Marcello, P., Swails, W., Kenler, A. *et al.*, Randomized phase I/II trial of a macrophage-specific immunomodulator (PGG-glucan) in high-risk surgical patients, *Ann. Surg.* 1994, *220*, 601 – 609.

[20] Bohn, J. A., BeMiller, J. N., (1→3)-beta-D-glucans as biological response modifiers: A review of structure-functional activity relationships, *Carbohyd. Pol.* 1995, *28*, 3 – 14.

[21] Hart, A. L., Stagg, A. J., Frame, M., Graffner, H. *et al.*, The role of the gut flora in health and disease, and its modification as therapy, *Aliment. Pharmacol. Ther.* 2002, *16*, 1383 – 1393.

[22] Saegusa, S., Totsuka, M., Kaminogawa, S., Hosoi, T., Candida albicans and Saccharomyces cerevisiae induce interleukin-8 production from intestinal epithelial-like Caco-2 cells in the presence of butyric acid, *FEMS Immunol. Med. Microbiol.* 2004, *41*, 227 – 235.

[23] Taylor, P. R., Brown, G. D., Reid, D. M., Willment, J. A. *et al.*, The beta-glucan receptor, dectin-1, is predominantly expressed on the surface of cells of the monocyte/macrophage and neutrophil lineages, *J. Immunol.* 2002, *169*, 3876 – 3882.

[24] Rice, P. J., Adams, E. L., Ozment-Skelton, T., Gonzales, A. *et al.*, Oral delivery and gastrointestinal absorption of soluble glucans stimulate increased resistance to infectious challenge, *J. Pharmacol. Exp. Ther.* 2005, *314*, 1079 – 1086.

[25] Grunebach, F., Weck, M. M., Reichert, J., Brossart, P., Molecular and functional characterization of human Dectin-1, *Exp. Hematol.* 2002, *30*, 1309 – 1315.

[26] Eckmann, L., Defence molecules in intestinal innate immunity against bacterial infections, *Curr. Opin. Gastroenterol.* 2005, *21*, 147 – 151.

[27] Wachtershauser, A., Stein, J., Rationale for the luminal provision of butyrate in intestinal diseases, *Eur. J. Nutr.* 2000, *39*, 164 – 171.

[28] Lia, A., Hallmans, G., Sandberg, A. S., Sundberg, B. *et al.*, Oat beta-glucan increases bile acid excretion and a fiber-rich barley fraction increases cholesterol excretion in ileostomy subjects, *Am. J. Clin. Nutr.* 1995, *62*, 1245 – 1251.

[29] Calmus, Y., Guechot, J., Podevin, P., Bonnefis, M. T. *et al.*, Differential effects of chenodeoxycholic and ursodeoxycholic acids on interleukin 1, interleukin 6 and tumor necrosis factor-alpha production by monocytes, *Hepatology* 1992, *16*, 719 – 723.

 