RESEARCH ARTICLE

Effects of mushroom-derived β-glucan-rich polysaccharide extracts on nitric oxide production by bone marrow-derived macrophages and nuclear factor-κB transactivation in Caco-2 reporter cells: Can effects be explained by structure?

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Mushrooms are known for their immune-modulating and anti-tumour properties. The polysaccharide fraction, mainly β-glucans, is responsible for the immune-modulating effects. Fungal β-glucans have been shown to activate leukocytes, which depend on structural characteristics of β-glucans. As edible mushrooms come in contact with the intestinal immune system, effects on enterocytes are also interesting. Our aim was to evaluate the effect of mushroom polysaccharide extracts varying in β-glucan structure on nitric oxide production by bone marrow-derived macrophages (BMMs) from mice and on nuclear factor-κB transactivation in human intestinal Caco-2 cells. We demonstrated that extracts from *Agaricus bisporus* stimulated nitric oxide production by BMM, whereas extracts from *Coprinus comatus* and spores of *Ganoderma lucidum* had only minor effects. Furthermore, extracts of *A. blazei* Murill and *Phellinus linteus* had no effect at all. Almost all mushroom extracts lowered nuclear factor-κB transactivation in Caco-2 cells. Structural analysis of *A. bisporus* compared with *A. blazei* Murill suggests that branching of the β-glucan chain is essential for immune-stimulating activity. In conclusion, extracts from *A. bisporus* activate BMM, without activating enterocytes. These characteristics make *A. bisporus* an attractive candidate as a nutritional compound to stimulate the immune response in depressed states of immunity.

Keywords:
Bone marrow-derived macrophages / Dietary / Immune response / Intestinal epithelial cells / Mushroom

1 Introduction

Mushrooms, which belong to the basidiomycetes and ascomycetes classes of the kingdom fungi, contain many different biologically active compounds, *e.g.* polysaccharides, small proteins, lectins, and polyphenols. The polysaccharides are mainly β-glucans that are present in the cell walls [1]. It is believed that these compounds have

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Abbreviations: BMMs, bone marrow-derived macrophages; **NF-κB,** nuclear factor-κB; **NO,** nitric oxide; **PMAA,** partially methylated alditol acetates; **ROS,** reactive oxygen species; **TNF-α,** tumour necrosis factor α

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immune-modulating effects [2–4]. Indeed, it has been reported that fungal β-glucans increase the phagocytic activity and the production of reactive oxygen intermediates [5], inflammatory mediators, and cytokines by leukocytes [6]. For *Pleurotus ostreatus* (Oyster mushroom), it was reported that a water extract from fruit bodies and mycelia played a role in increasing the production of reactive oxygen species (ROS) from neutrophils and has also additional immune-modulating properties involving all immunocompetent cells [7].

As part of the human diet, compounds derived from digested edible mushrooms come in contact with enterocytes, which are located at the frontline of the intestinal immune system. Enterocytes have to discriminate between harmful and harmless antigens. To perform this task they play an important role in orchestrating the intestinal immune response and can induce an inflammatory response if necessary. Therefore, it is likely that mushrooms might also affect the immune response of enterocytes. It has been demonstrated that intestinal macrophages [8] and enterocytes (presumably M-cells) [9, 10] can take up and distribute β-glucans after oral intake. However, one study suggested that β-glucans from a fungal extract did not enter the blood as the effect *in vivo* was strongly diminished compared with *in vitro* [11]. To examine whether the mushroom polysaccharide extracts have an effect on enterocytes we used a nuclear factor-κB (NF-κB) reporter Caco-2 cell line. NF-κB is a transcription factor that plays a key role in the immune response. In inactive form it is present in the cytoplasm bound by its inhibitor IκB. Upon stimulation IκB is degraded by IκB kinase and NF-κB is free to move to the nucleus where it can bind to response elements of NF-κB target genes. One of the consequences of NF-κB activation is activation of the numerous NF-κB target genes, which results in the production of cytokines like tumour necrosis factor α (TNF-α) and ROS. Responsiveness of macrophages towards β-glucan treatment has already been reported by many different groups [12–15]. More into detail, β-glucans have been classified as biological response modifiers, which can influence the activity of macrophages [12, 14, 15]. For that reason bone marrow-derived macrophages (BMMs) have been used by others to examine the systemic immune-modulating properties of β-glucans. For this nitric oxide (NO), which is produced by the NF-κB-regulated enzyme NO synthase, was chosen as one of the outcome parameters in the BMM experiments as fungal β-glucans have been reported to modulate NO production [16–18].

β-Glucans consist of a backbone formed by linear β(1→3)-, β(1→4)-, or β(1→6)-linked glucose molecules, which in addition can contain branches. The type of linkages of the backbone and branches differs for the different β-glucans. For example, it has been suggested that β-glucans from yeast and fungi consist of β(1→3)-linked glucopyranosyl units with β(1→6)-linked side chains, whereas β-glucans from oat and barley are composed of unbranched β(1→3) and β(1→4) linkages [6, 19, 20]. The activity of β-glucans may depend on their structural properties, such as polymer length, degree of branching, tertiary structure, and molecular weight [21]. Besides the structure, the method of isolation can also affect the activity of β-glucans [1].

The aim of this study was to investigate the effect of several β-glucan-rich mushroom polysaccharide extracts, which were *a priori* based on the literature expected to vary in β-glucan structure, on NO production by BMMs from mice as well as on NF-κB transactivation in a human small intestinal enterocyte-like cell line, the Caco-2 cell. As besides the structure the method of isolation can also affect the immune-modulating activity of β-glucans, we also evaluated the effect of the mushroom extract with the highest *in vitro* activity in the BMM cells, *e.g.* the extract from *Agaricus bisporus*, when isolated with a different methodology. Additionally, we tried to find a structure–-response relationship by evaluating some structural characteristics of two extracts with opposite effects, *e.g.* the extracts from *A. bisporus* and *A. blazei* Murill. We hypothesized that the branched β-glucans are the most potent immune-stimulating extracts.

2 Materials and methods

2.1 Preparation of mushroom polysaccharide extracts

Different mushroom polysaccharide extracts were isolated and used for BMM and Caco-2 NF-κB reporter cell stimulations. Extracts were isolated from spores (A) and fruit body tissue (B) of *Ganoderma lucidum*; from different parts of the *A. bisporus*, *e.g.* the caps and stems; and from *A. blazei* Murill, *Coprinus comatus*, and *Phellinus linteus*. In addition, α-glucans were extracted from the stems of *A. bisporus* to distinguish between α-glucan and β-glucan effects. Semi-purified polysaccharides were obtained from fruiting body material by hot water extraction followed by ethanol precipitation as described earlier [5]. A final solution was made in PBS at 900 μg/mL, which was then heated for 1 h at 100°C and stored at 4°C. The extracts will be further referred to as fruit body polysaccharide extracts. α(1→4) Glucan was obtained from *A. bisporus* stripes by passage of the polysaccharide extract over a diethylaminoethyl-cellulose column. The colourless non-binding polysaccharide has a MW > 200 kDa, which can be completely degraded by α-amylase, and is characterized as an α-glucan [5].

A. bisporus mushroom polysaccharide extract was also isolated from culture mycelium by centrifugation and filtration. To this extract four volumes of ethanol were added and precipitation was allowed to proceed overnight at 4°C. The resulting precipitate was redissolved in water. To obtain the extract, the above-described procedure was repeated twice. The resulting fluid was sterilized by a 0.45 μm filter and adjusted to a concentration of 900 μg/mL and further called culture mycelium extracts.

2.2 Chemical analysis of glucans from *Agaricus* species

To try and explain the physiological effects due to differences in structure, we determined the monosaccharide composition and analysed the glycosidic linkages of *A. bisporus* and *A. blazei* Murill mushroom polysaccharide extracts.

2.2.1 Monosaccharide composition

To evaluate the monosaccharide composition, a 5 μL aliquot of the glucan solution or suspension was diluted in 0.3 mL 2 M TFA and hydrolyzed overnight at 100°C. Solutions were evaporated to dryness at 40°C under an N_2 stream. Residual TFA was removed by two evaporation cycles in 0.5 mL methanol, as earlier. The final residue was dissolved in 0.5 mL water and analysed for monosaccharides on a Dionex HPLC, equipped with pulsed amperometric electrochemical gold detector. A PA1-column (250 × 4 mm), fitted with a PA-1 guard column (10 × 32 mm), was used as the stationary phase and 0.020 M NaOH as the mobile phase at 1.0 mL/min (isocratic elution). Quantification of monosaccharides was performed on the basis of external standards.

2.2.2 Linkage analyses *via* GCMS of partially methylated alditol acetates

To determine the distribution of glycosidic linkages in the polysaccharides GCMS analysis of partially methylated alditol acetates (PMAAs) derived from these compounds was performed. Permethylation was performed according to one of the protocols described in Kang *et al.* [22] where 0.7% water was added during the incubation. Permethylated products were separated by phase partitioning after adding 0.5 mL cold (4°C) chloroform and 2.5 mL cold water. The chloroform phase was washed three times with 2.5 mL water to remove all NaOH, evaporated to dryness, and hydrolysed overnight in 0.3 mL 0.5 M TFA at 100°C. Further processing to PMAAs was performed as described in Ref. [23]. PMAAs were quantified by GC-MS using a Hewlett Packard HP 5890 gas chromatograph connected to a HP 5972A mass spectrometer, which was equipped with a ZB-5 column (30 m, id 0.25 mm, layer thickness 0.25 μm), run at the following gradient: 3 min 170°C, 2°C/min to 210°C, then 10°C/min to 230°C, and a final time of 4 min.

2.3 BMM stimulation

BMMs were isolated from the tibiae and femurs of C57BL/6 mice. Cells were cultured in 15 cm bacterial plastic petri-dishes in standard RPMI with L-glutamine and HEPES (Invitrogen, Breda, The Netherlands), 10% heat-inactivated fetal calf serum (Bodinco BV, Alkmaar, The Netherlands), and 100 U/mL penicillin streptomycin (Invitrogen) with the addition of 15% L929 cell-conditioned medium kindly provided by Danielle Curfs (Department of Molecular Genetics, Maastricht University, The Netherlands). After 1-wk differentiation, cells were detached with lidocaine (Sigma Chemicals, St Louis, MO, USA) and plated at a concentration of 1×10^6 cells/mL in a final volume of 0.5 mL. Cells were primed with 100 U/mL IFN-γ during 24 h to obtain a maximal effect on NO production, as described earlier [24]. Then the medium was refreshed and cells were incubated with the mushroom polysaccharide extracts in a concentration range of 50–200 μg/mL for 24 h. These concentrations are physiological as we reported earlier that the β-glucan concentration in the 24 h pooled fecal water obtained from ileostomic patients who consumed an oat β-glucan-enriched diet varied between 120 and 180 μg/mL [25]. Besides mushroom polysaccharide extracts we also evaluated the effects of cellulose, a linear molecule that consists of β(1→4) linkages (Sigma-Aldrich, Steinheim, Germany) on BMM-derived NO production to further substantiate our assumptions regarding a possible structure–effect relationship. Supernatants from the BMM were removed after 24 h incubation and NO concentrations were determined. To evaluate whether the effects of the mushroom polysaccharide on NO production were mediated by the β-glucan receptor dectin-1, we used the dectin-1 inhibitor laminarin. For this, cells were incubated with 250 or 500 μg/mL laminarin (Sigma Chemicals) 1 h before stimulation with 200 μg/mL *A. bisporus* stems, *A. bisporus* stems α-glucans, *A. blazei* Murill, and cellulose.

2.4 Measurement of NO and TNF-α concentrations

NO concentrations in the supernatants of BMM were determined as total nitrite by the Greiss method [26]. In short, 50 μL supernatant was combined with 50 μL Greiss reagents (2.5% v/v phosphoric acid, 0.1% w/v naphthylethylene diamine dihydrochloride, and 1.0% w/v sulphanilamide in distilled water). After incubation for 10 min at room temperature, absorbance was determined at 540 nm. Sodium nitrate (Sigma Chemicals) was used to prepare a standard curve (0–200 mM). Besides NO production we also determined TNF-α production by BMM after stimulation with *A. bisporus* stems, *A. bisporus* stems α-glucan, and *A. blazei* Murill preincubated with 500 μg/mL laminarin. TNF-α concentrations were measured as described in Ref. [27].

2.5 Intestinal cell culture

We also determined the effect of all mushroom polysaccharide extracts on enterocytes. For this, we used the human cell line Caco-2, which was obtained from the American Tissue Type Collection. Caco-2 cells were cultured in DMEM (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum

(Invitrogen), 1% penicillin streptomycin (Invitrogen), 1% sodium pyruvate, and 1% non-essential amino acids (Invitrogen). Cells were cultured at 37°C in a 5% CO_2 humidified atmosphere, refreshed every second day, and separated by trypsin-0.03% EDTA (Gibco BRL, Gaithersburg, MD) when they had reached 70–90% confluence.

2.6 Stable transfection of NF-κB in Caco-2 cells

To evaluate the effects of the various mushroom-derived polysaccharide extracts on transcriptional activity of NF-κB, a stable NF-κB reporter Caco-2 cell line was created. This cell line was chosen as we have previously shown immune-stimulating effects of oat β-glucan fecal water in Caco-2 cells [25]. The 6κB-TK-luciferase (NF-κB reporter) plasmid and neomycin-resistance plasmid were kindly provided by Dr. R. C. Langen (Department of Pulmonology, Maastricht University, The Netherlands). Cells were transfected using Lipofectamine 2000 (Invitrogen) according the manufacturer's instructions. Positive clones were selected by culturing with geneticin (1 mg/mL). For the experiments, Caco-2 NF-κB reporter cells were plated in 24-well plates at an initial density of 0.3×10^6 cells/mL in a total volume of 0.5 mL and used for stimulation experiments when they had reached 70–90% confluence. Culture medium was replaced by fresh medium containing different mushroom polysaccharides at physiological concentrations in combination with an inflammation-inducing cytokine cocktail consisting of IFN-γ (100 U/mL) and IL-1β (50 U/mL). After 3 h of incubation, cells were lysed in luciferase lysis buffer (Promega, Madison, WI, USA) and stored at −80°C. Luciferase activity was measured according to the manufacturer's instructions (Promega) and expressed relative to total protein (Bio-rad assay; Bio-rad, Hercules, CA, USA).

2.7 Statistical analysis

All parameters are presented as means ± SD and were analysed for statistical differences by ANOVA. When a significant difference in concentration was found, the four concentrations were compared pairwise using a Tukey *post hoc* test for multiple comparisons of observed means. Differences were considered significant at $p < 0.05$ (corrected for four groups by SPSS). All statistical analyses were performed using SPSS 15.0 (SPSS, Chicago, IL, USA).

3 Results

3.1 Effects of mushroom polysaccharide extracts on NO and TNF-α production by BMM

As demonstrated in Fig. 1, extracts isolated from *A. bisporus* fruit bodies significantly increased NO production by BMM.

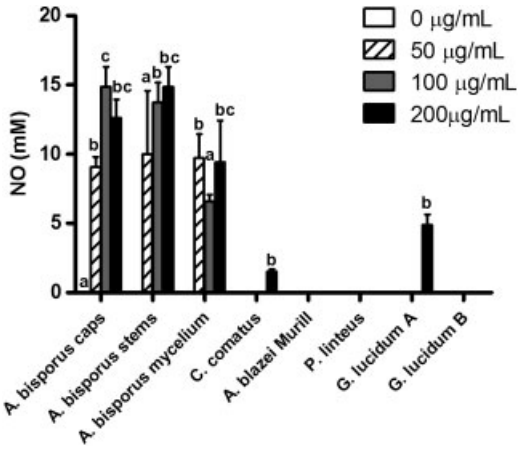

Figure 1. Increased NO production by IFN-γ (100 U/mL) primed BMM after 24 h stimulation with *A. bisporus* mushroom polysaccharide extracts. Extracts isolated from *C. comatus* and *G. lucidum* mushroom polysaccharide extracts had minor effects. Values are means and SD of duplicate cultures. Experiments have been conducted three times and a representative example is shown. Bars with different superscripts differ ($p < 0.05$). Superscript 'a' refers to other mushroom polysaccharide extracts.

These effects were almost identical for extracts isolated from caps and stems. In addition, NO production was also increased ($p < 0.05$) when BMM was incubated with mycelium extract isolated from *A. bisporus* indicating that the effect was independent of the method of culturing and isolation. Interestingly, the effects on NO production were not observed for all fruit body extracts. Spore extracts from *G. lucidum* and *C. comatus* also stimulated NO production but only significant at the highest concentration and not as prominent as extracts from *A. bisporus*. The polysaccharide extracts from *A. blazei* Murill, *P. linteus*, and *G. lucidum* fruiting bodies had no NO-inducing activity.

As the cell wall of mushroom consists of a large part of the polysaccharide β-glucan, it is tempting to suggest that these polysaccharides increased NO production. Therefore, to test whether effects of *A. bisporus* were β-glucan-specific, we also evaluated effects of *A. bisporus* derived α-glucans from the stems on BMM-derived NO production. In line with β-glucans isolated from *A. bisporus*, *A. bisporus* stem α-glucans dose-dependently increased NO production to a maximum of 12 mM (Fig. 2). However, the maximum NO concentration that was reached was only 50% of the concentration observed after incubation with the *A. bisporus* polysaccharide extract containing merely β-glucans. Cellulose consisting of only β(1→4) linkages had no effect on NO production by BMM (Fig. 2). For TNF-α production we observed a comparable dose–response relationship as for NO (Fig. 3).

To evaluate the potential involvement of the β-glucan receptor dectin-1 in the observed NO production by BMM,

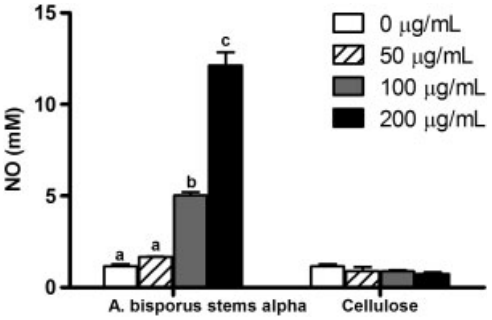

Figure 2. Mushroom polysaccharide extracts isolated from *A. bisporus* alpha dose-dependently increased NO production by IFN-γ (100 U/mL) primed BMM after 24 h stimulation, whereas cellulose had no effect. Values are means and SD of duplicate cultures. Bars with different superscripts differ ($p<0.005$).

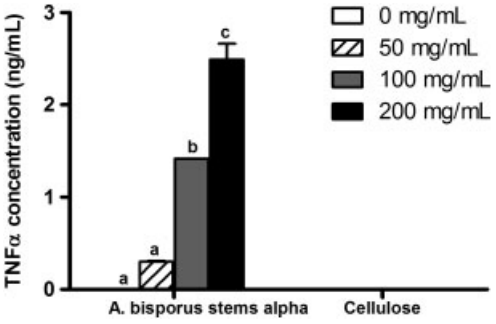

Figure 3. Mushroom polysaccharide extracts isolated from *A. bisporus* alpha dose-dependently increased TNF-α production by IFN-γ (100 U/mL) primed BMM after 24 h stimulation, whereas cellulose had no effect. Values are means and SD of duplicate cultures. Bars with different superscripts differ ($p<0.001$).

the dectin-1 inhibitor laminarin was used. As shown in Figs. 4 and 5, laminarin had no effect on the *A. bisporus* stems β-glucan-induced NO and TNF-α production. Comparable with the results described earlier, also in the presence of laminarin, *A. bisporus* stems α-glucan stimulated the NO and TNF-α production to 50% beyond the level of *A. bisporus* stems β-glucans, whereas extracts isolated from *A. blazei* Murill and cellulose again showed no effect (Figs. 4 and 5).

3.2 Effects of *A. bisporus* stems mushroom polysaccharide extracts on NF-κB transactivation in enterocytes

To evaluate whether the polysaccharide extracts isolated from the edible mushroom *A. bisporus* and the other mushrooms also modulated the immune response of enterocytes, we measured NF-κB transactivation in our Caco-2 NF-κB reporter cell line. Figure 6A demonstrates that the effects of extracts isolated from the fruit bodies were highly variable; however, almost all extracts lowered the

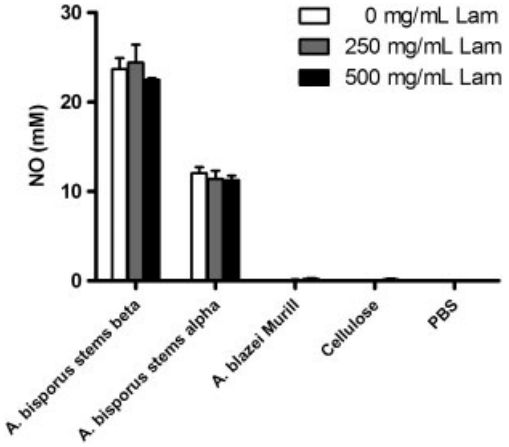

Figure 4. Dectin-1 inhibitor laminarin was unable to inhibit the increased NO production by BMM caused by 200 μg/mL *A. bisporus* mushroom polysaccharide extracts. Values are means and SD of duplicate cultures. Experiments have been conducted two times and a representative example is shown.

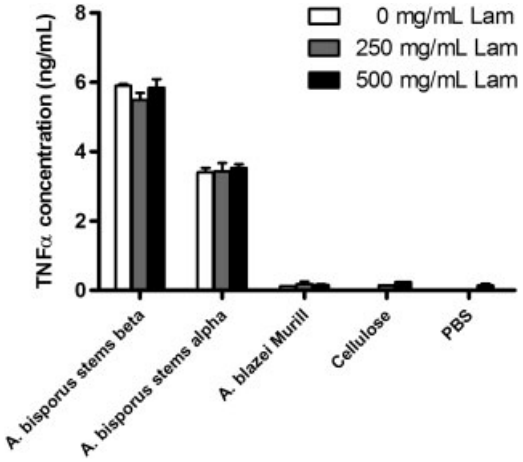

Figure 5. Dectin-1 inhibitor laminarin was unable to inhibit the increased TNF-α production by BMM caused by 200 μg/mL *A. bisporus* mushroom polysaccharide extracts. Values are means and SD of duplicate cultures. Experiments have been conducted two times and a representative example is shown.

transactivation of NF-κB in Caco-2 cells as compared with buffered saline. This decreased NF-κB transactivation was most obvious after incubation with the highest concentration of the extracts, whereas at 100 μg/mL some extracts even showed an increased NF-κB transactivation. The magnitude of the effect differed between the mushroom extracts. The extract isolated from *A. blazei* Murill and *C. comatus* fruit bodies showed the most pronounced decrease in NF-κB transactivation (35.3 and 41.4% ($p = 0.074$) at the highest concentration, respectively), whereas *P. linteus* had no effect at all. Furthermore, extracts from both spores and fruiting bodies of *G. lucidum* reduced NF-κB transactivation at 50 and 200 μg/mL. Finally, extracts

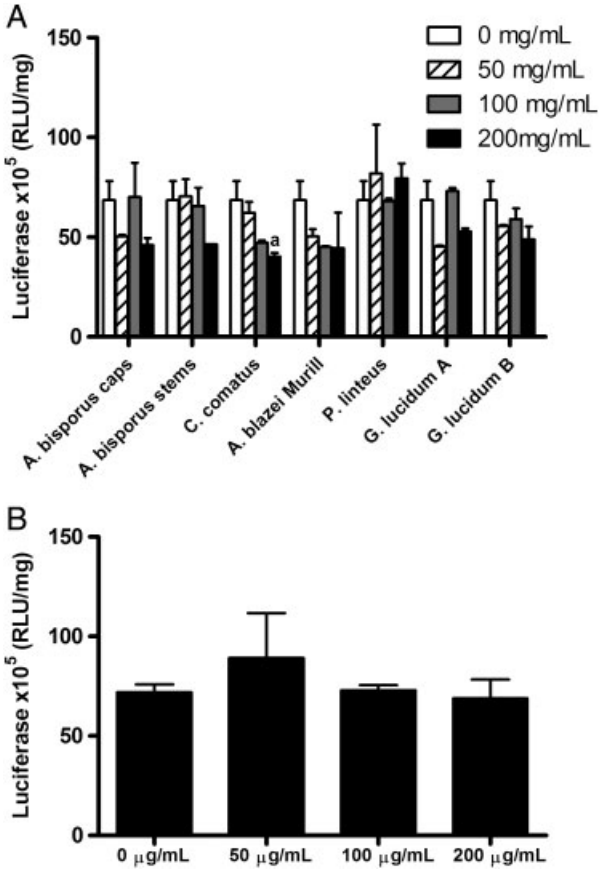

Figure 6. (A) Most of the different mushroom polysaccharide extracts decreased NF-κB transactivation by Caco-2 NF-κB reporter cells after 3 h stimulation in presence of IL-1β (100 U/mL) and IFN-γ (50 U/mL). Values are means and SD of duplicate cultures. Experiments have been conducted three times and a representative example is shown. Superscript 'a' refers to $p = 0.074$, 200 μg/mL *versus* control. (B) NF-κB transactivation by Caco-2 NF-κB reporter cells after 3 h stimulation with *A. bisporus* isolated from the culture medium in the presence of IL-1β (100 U/mL) and IFN-γ (50 U/mL) is increased. Values are means and SD of duplicate cultures. Experiments have been conducted three times and a representative example is shown.

isolated from two different parts of *A. bisporus* (stems or caps) showed similar effects at the highest concentration (reductions of 32 and 33%, respectively). Surprisingly, in contrast to extracts isolated from the *A. bisporus* fruiting bodies, extracts isolated from *A. bisporus* culture medium did not lower NF-κB transactivation (Fig. 6B).

3.3 Chemical analysis of glucans from *A. bisporus versus A. blazei* Murill

As the activity of β-glucans depends on the chemical structure, we determined the monosaccharide composition and linkage type to explain the differences in immune-modulating properties of *A. bisporus* and *A. blazei* Murill. The

Table 1. Monosaccharide composition of *A. bisporus* and *A. blazei* Murril as determined by HPLC

Monosaccharide	*A. bisporus*	*A. blazei* Murill
Glucose	93.3	57.7
Galactose	2.9	27.7
Mannose/xylose	0.8	7.3
Fucose	nd	4.0
Rhamnose	nd	1.2
Arabinose	nd	1.2
GLcNH2	nd	0.9
Fructose	nd	0.7

nd, not detected.

Table 2. Linkages of *A. bisporus* and *A. blazei* Murril as determined *via* GCMS of PMAAs

Linkage type	*A. bisporus*	*A. blazei* Murill
Terminal glucose	9.5	10.8
β(1→3)	3.5	5.1
β(1→4)	85.5	43.3
β(1→6)	nd	40.8
β(1→3, 4) branch	1.6	nd
β(1→3, 6) branch	nd	nd
β(1→4, 6) branch	nd	nd

nd, not detected.

monosaccharide compositions of *A. bisporus and A. blazei* Murill are presented in Table 1. The polysaccharides of *A. bisporus* consisted of mainly glucose (96.3%) with some minor proportions of galactose (2.9%) and xylose (0.8%). This polysaccharide can thus be considered as consisting of mainly glucans. *A. blazei* Murill polysaccharide was more complex as it contained in addition to glucose (57.7%) considerable proportions of galactose (27.7%); mannose and xylose (not separated, together 7.3%); fucose (4%); and some rhamnose, arabinose, fructose, and GlcNH2 (each about 1%). $GlcNH_2$ was probably present in the native oligosaccharide as GlcNAc, which was degraded by TFA hydrolysis.

In addition to the monosaccharide composition, we also measured the glycosidic type of linkage and branching. *A. bisporus* glucan contained predominantly 1→4 and a few 1→3 linkages, while no 1→6 linkages were measured (Table 2). In agreement with the presence of 1→3 and 1→4 linkages, 1→3,4-branching glucose residues were also observed at about 1.5%. The relative high number of terminal glucose residues and low number of branch points indicate a short-chain molecule. The high proportion of 1→4 glucan suggests that the main chain contains predominantly this type of linkage.

For *A. blazei* Murill the linkage was different as more than 80% of the glucose residues in the glucan fraction were linked by either 1→4 or 1→6 linkages at a ratio of about 1:1, while a small proportion of 1→3 glucosidic linkages were also

observed and with no branch points. The observations from this PMAA analysis may be interpreted by either a single main chain with alternating 1→4 and 1→6 linkages or by a mixture of more glucan chains with a single linkage type.

4 Discussion

Fungal β-glucans are known for their immune-modulating and tumour growth-inhibiting effects [2–4]. It has been suggested that the polysaccharides present in mushrooms are the most likely candidates for these effects. As edible mushrooms are present in our daily diet, effects of these mushrooms on (intestinal) immune responses are of potential interest for optimizing the functioning of our immune system. We here show that extracts isolated from *A. bisporus* – either from caps and stems of *A. bisporus* fruit bodies or from *A. bisporus* mycelium – stimulated the production of NO (a NF-κB target gene) of BMM. Furthermore, purified α-glucan from the stems of *A. bisporus* dose-dependently stimulated the NO production also, although the maximum effect observed was only 50% of that achieved with *A. bisporus* β-glucan. When using cellulose, consisting of only β(1→4) linkages, no NO was produced. All results were identical if TNF-α production by BMM was analysed instead of NO. Immune-modulating effects of β-glucans are generally accepted, whereas the effects for α-glucan are debated. However, the fact that *A. bisporus* α-glucans stimulated BMM to a lesser extent as compared with *A. bisporus* β-glucans at least illustrates that effects are glucan-type dependent. Interestingly, effects were not identical for all mushroom extracts. *C. comatus* and *G. lucidum* A extracts had only minor effects on NO concentrations as compared with the effects of *A. bisporus*, whereas *A. blazei* Murill and *P. linteus* had no effect at all. Earlier, immune-stimulating effects of mycelium and fruit body extracts from *A. blazei* Murill, although not for all fractions, have been reported [13, 28]. It has been shown that the method of isolation and chemical modification can influence the activity of β-glucans, which may explain the difference in results between the studies [1, 3, 29]. The largest difference in effects on BMM behaviour was found for *A. bisporus* and *A. blazei* Murill, in which *A. bisporus* was the most potent immune-stimulating extract, whereas *A. blazei* Murill extract showed almost no effect. To explain these differences in activating potential we determined some structural characteristics of both extracts and tried to define a sort of structure–effect relationship. Chemical analysis demonstrated that the polysaccharide from *A. bisporus* consists of mainly glucose, whereas that from *A. blazei* Murill is a mixture of different monosaccharides. This may suggest that a β-glucan almost exclusively composed of glucose exerts a greater immune-stimulating activity as compared with glucans containing other monosaccharides also. However, cellulose consisting solely of glucose molecules has no NO-inducing activity on BMM. This implies that other factors besides monosaccharide composition play a role. A second pronounced structural difference between both extracts we observed was the type of linkages. *A. bisporus* was mostly composed of 1→4 linkages and some 1→3 linkages, while *A. blazei* Murill contains 1→4, 1→6, and some 1→3 linkages. It has been reported that 1→3 linkages are essential for immune-modulating activity and both extracts contain approximately the same amount of 1→3 linkages so this could not explain the difference in immune-modulating properties. Moreover, *A. bisporus* is in our hands free of 1→6-linkages, although earlier reports describe the presence of such linkages in glucans from this fungus [30]. The absence of 1→6 linkages in our extract may be due to the origin and developmental stage of the fungal tissue used for the isolation of glucans. Moreover, our method of extraction mainly isolates the mucilage layer of the cell wall, which has been reported to consist mostly of 1→4 linked polysaccharides with minor portions of 1→6 linkages [31]. In contrast, hot water extracts of only lamellae consist of polysaccharides with relatively high numbers of 1→6 linkages [32], whereas polysaccharide extracts made with KOH instead of hot water consist of mainly 1→3 and 1→6 linkages. Despite the fact that our *A. bisporus* lacks 1→6 linkages, the immune-stimulating activity as described for other *A. bisporus* extracts remains [13]. Furthermore, *A. blazei* Murill has no branch points, which indicates a single chain molecule, where *A. bisporus* is branched with a few 1→3 side chains. It is generally believed that branches are important for immune-modulating activity of glucans [29], so this may explain the different immune-stimulating activities of *A. bisporus* and *A. blazei* Murill. This is further supported by the observation that oat β-glucan, which also lacks branches, showed comparable results on both BMM-derived NO and TNF-α as *A. blazei* Murill (data not shown). Oat β-glucan is composed of 1→3 and 1→4 linkages and the difference compared with *A. blazei* Murill is the absence of 1→6 linkages. However, this is not essential for immune-stimulating activity as these linkages are also absent in *A. bisporus*. Moreover, unbranched cellulose has no NO-inducing activity on BMM, although this could also be explained by the fact that this β-glucan consists only of 1→4 linkages. Overall, we conclude from this study that 1→3 branches are essential for immune-stimulating activity of mushroom polysaccharides as this is the main difference between *A. bisporus* and all other glucans tested. Nevertheless, we must remark that immune-modulating effects of other compounds present in the mushroom polysaccharide extracts, such as polyphenols, cannot be excluded. Unfortunately, we do not have detailed information concerning the type or concentration of polyphenols as present in the mushroom polysaccharide extracts.

There seems to be a general awareness that the β-glucan receptor dectin-1 mediates the β-glucans-induced immune-stimulating effects of macrophages [6]. However, in our hands, dectin-1 seems not to be involved in the observed NO production after *A. bisporus* by BMMs, as preincubation with

the dectin-1 antagonist laminarin did not affect the response. This implies that the mushroom polysaccharide extracts exert their effect *via* another mechanism or receptor. This is in agreement with Wei *et al.* [5], who found no competing effects of laminarin on ROS production after addition of *A. brasiliensis* and *G. lucidum* by peripheral blood mononuclear cells. Other potential candidate receptors for the observed effects that have been suggested in this perspective are CR3, lacosylceramide, scavenger receptors, Toll-like receptor 2 or 4 [5, 33].

Since the mushroom polysaccharide extracts isolated from the edible *A. bisporus* showed the most potent immune-stimulating effect on BMM, we were also interested in its immune-modulating effects on enterocytes as the intestine is the place where enterocytes have to discriminate between potential harmful pathogens and vulnerable nutrients. These cells are located at the frontline of the intestinal immune system and orchestrate the immune response. Therefore, it has been suggested that oral administration of mushroom extracts is an easy way of increasing immunological tolerance, anti-tumour effect, and innate immunity against intracellular pathogens. In this respect, it has been shown that the consumption of mushroom derivatives can influence the (intestinal) immune response in mice. When extracts isolated from *Lentinula edodes* were dispersed with lecithin micelles and administered to mice, the number of intestinal epithelial lymphocytes in the small intestine increased as well as TNF-α and IL-2 in serum [23]. Furthermore, mice fed with water soluble extracts isolated from *G. lucidum* and *A. blazei* Murill showed prolonged survival time after x-irradiation and the small intestinal crypt survival was increased [34], which is another example of immune modulation through consumption of mushrooms. To evaluate the effects of the mushroom polysaccharide extracts, and especially those from *A. bisporus*, we used NF-κB transactivation as outcome measurement since this transcription factor plays a central role in the immune response. We demonstrated that the different mushroom polysaccharide extracts isolated from the fruit body tissue lowered NF-κB transactivation in Caco-2 NF-κB reporter cells, including extracts from the highly consumed *A. bisporus*. Although almost all extracts showed a decreased luciferase activity, the effects were highly variable between the extracts, which may be caused by biological diversity in our cell model or different threshold concentrations. However, when the extract from *A. bisporus* was isolated from the mycelium instead of the fruit body, no reduced NF-κB transactivation was seen. The contrast can be explained by the method of isolation, which has been described to influence the activity of β-glucans [1].

Thus, the observed effects of the mushroom polysaccharide extracts, especially those isolated from *A. bisporus*, on transactivation of NF-κB in Caco-2 NF-κB reporter cells were opposite to the activating effects seen in BMM. This discrepancy may be explained by the fact that intestinal cells are constantly exposed to pathogens from the intestinal lumen where they mostly dampen the local immune response to prevent severe intestinal inflammation, which is reflected by a decreased transactivation of the central inflammation marker NF-κB. However, systemic immune cells like BMM are not continuously confronted with pathogens and if so an effective response is desirable. Moreover, we must remark that the macrophage data were obtained by using mouse macrophages. However, similar effects of human and mice primary macrophages on pro-inflammatory cytokine production after exposure to zymosan, a crude β-glucan extract, have been reported [35].

In summary, we demonstrated that effects of mushroom polysaccharides on NO production by BMM depend on structural characteristics of the polysaccharides. *A. bisporus* showed a strong immune-stimulating effect on BMM, whereas *A. blazei* Murill has almost no effect. In contrast to the exclusive activating effects of *A. bisporus* in BMM almost all mushroom extracts lowered NF-κB transactivation in Caco-2 enterocyte cells. Altogether, the edible mushroom *A. bisporus* seems an interesting nutritional compound in situations where the activity of the immune system needs to be stimulated, as *A. bisporus* activates macrophages without activating enterocytes. This might, for example, be beneficial in depressed states of immunity. The functionality of this assumption needs to be confirmed in studies especially designed for this research question.

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