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Enhanced Anti-Inflammatory Effects by Glucans Extracted from the Stalks of *Pleurotus Eryngii* Grown In Substrates Containing Olive Mill Waste

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Abstract

We recently demonstrated that the mushroom *Pleurotus eryngii* grown on a substrate containing increasing concentrations of olive mill solid waste (OMSW) contain greater glucan concentrations as a function of OMSW content. Treatment of rat Intestinal epithelial cells (IEC-6) transiently transfected with Nf-κB fused to luciferase demonstrated that glucans extracted from *P. eryngii* stalks grown on 80% OMSW down regulated TNF-α activation. Glucans from mushrooms grown on 80% OMSW exerted the most significant reducing activity of nitric oxide production in lipopolysaccharide (LPS) treated J774A.1 murine macrophages. The isolated glucans were tested in vivo using the Dextran Sodium Sulfate (DSS) induced colitis in C57Bl/6 mice and found to reduce the histology damaging score resulting from DSS treatment. Expressions of various intestinal cytokines were efficiently down regulated by treatment with the fungal extracted glucans. We conclude that the stress-induced growing conditions exerted by OMSW induce production of more effective anti-inflammatory glucans in *P. eryngii* stalks.

Keywords: Glucans; Inflammation; Inflammatory Bowel Disease; *Pleurotus eryngii*

Introduction

Pleurotus eryngii, is the largest species belonging to the edible oyster mushroom genus and widely consumed in Europe, Western Asia, America, Africa and India. The nutritional advantages on consumption of this mushroom are well documented [1, 2]. The beneficial effects exerted by *P. eryngii* are mediated at least partially by the glucan content of this edible mushroom. *P. eryngii* are rich in both α and β-glucans [3-5]. Glucans from edible mushrooms have been repeatedly shown to exert a strong regulatory effect of the immune system [3, 6-8]. The regulation of the immune system by glucans appears to be partially mediated by direct activation of the innate immune system, mainly following interaction with dendritic cells and macrophages [4, 9].

Glucans activate a biological response, partially, by specific binding to lectin-binding site of complement receptor type III (CR3 (CD11b/CD18)) on immune effector cells. Dectin-1, also known as β-glucan receptor, recognizes β-(1, 3) linkage and plays an important role in anti-fungal infection. Dectin-1 is expressed on natural killer cells, dendritic cells, macrophages, monocytes, neutrophils and T cells. The dectin-1 activation signal mechanism promotes innate immune responses through activation of phagocytosis, ROS production mediated by triggering the transcription factor nuclear factor-κB (NF-κB) to induce cytokine and chemokine synthesis [5, 10]. Nitric oxide is a small free radical secreted by activated macrophages and functions as a well-known inflammatory mediator. It has been previously demonstrated that β-glucans can significantly reduce NO production in activated macrophages [2, 9, 11-14].

Nuclear factor-κB is a protein considered to participate in one of the most well-established proinflammatory signaling pathways and thus controls the expression of many inflammatory cytokines and chemokines; its expression and activation provides an important step in the inflammatory process including the intestinal inflammatory pathway [15-17].

Olive Oil Mill Solid Waste (OSMW) is the dry solid fraction that remains after water is removed from the waste product of olive oil production. We have recently demonstrated that increasing the fraction of OSMW in the growth substrate of *P. eryngii* induces a significant increase in the amount of total glucans and especially α-glucans produced by the edible mushroom. We also showed that the distribution is not equal between the different parts of the mushroom and that the *P. eryngii* mushroom stalks accumulated more glucans than caps [18]. Our findings show a dose response-dependent increase in glucan production as a function of % OSMW increases in the substrate.

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Inflammatory Bowel Diseases (IBD) are divided into two major types; Crohn's disease and ulcerative colitis. Both diseases exhibit an overactive immune response in the gastrointestinal tract. IBD are chronic diseases causing a dramatic reduction of the quality of life and substantial health costs. Treatment of IBD can be targeted by non-specific immunosuppressive therapies-steroids, antibiotics- or alternatively by biological therapies. The latter consists of targeting specific proinflammatory mediators including Tumor Necrosis Factor (TNF) and cytokines [19]. Such treatments are not always effective and sometimes include severe adverse side effects.

The aim of the present study was to investigate the anti-inflammatory effect of glucans extracted from stalks of *P. eryngii* grown of on substrate supplemented with 20% OSMW as compared to 80% OSMW. We assessed the effect of the extracted glucans *in vitro* on both intestinal epithelial cells and cells of the immune system and *in-vivo* using the Dextran Sodium Sulfate (DSS) induced colitis in mice.

Materials and Methods

Mushroom Growth Conditions

P. eryngii mushrooms were grown as recently described at the Matityahu Experimental Farm (Upper Galilei, Israel) on a sterilized mixture of eucalyptus sawdust and OMSW [18]. OMSW is the solid fraction from 3 phase olive mill. After olive mill wastewater was discharged OMSW was added at concentrations from 20% (w/w) and 80% (w/w). The mixture was brought to a water content of 51% and packed into 15 liter polypropylene bags, containing a microporous filter, 2 kg wet substrate/bag. The bags were autoclaved at 121 °C for 1 h, closed and cooled to 25 °C for inoculation with spawn. Incubation was allowed for 14–21 days at 25°C. To allow fruiting, the bags were opened and the temperature was reduced to 16 °C with a relative humidity of 90%, 12 h daily light and CO_2 concentration of 600-800 ppm. *P. eryngii* were dried, milled and filtered through a 1.0 mm screen using a Retsch centrifugal mill preparations unit.

Preparation of Glucans

Powdered dried mushrooms mill of *P. eryngii* stalks were extracted as recently described [18]. In short, 100 g of mill were suspended in 1,000 ml dH_2O and heated to 121 °C for 30 min (in autoclave). The extract was then centrifuged at 13,000 × g at 10 °C for 15 min. Ethanol was added to the supernatant to a final concentration of 67% (v/v), and the mixture was stored overnight at -20 °C. The float was taken out, lyophilized and glucan content analyzed on the solubilized dried lyophilized material. For in-vitro assays lyophilized glucans were solubilized in sterile ddH_2O at 80°C for 1h with vortex every 15 min to final concentration of 10 mg/mL. Solubilized samples were then autoclaved as before.

Glucans Analysis

Assessment of glucan concentrations in the different glucan extracts from *P. eryngii* grown on either 20% or 80% OSMW was determined using a mushroom and yeast specific β-glucan kit (Megazyme International, Wicklow, Ireland) based on a colorimetric reaction using the recently described method [18]. The absorbance of the resulting color complex was measured using a spectrophotometer (Synergy 2, Multi-Mode Reader, BioTek, Winooski, VT, USA) at 510 nm. Total glucan (% w/w), α-glucan (% w/w) and β-glucan content (% w/w) were measured as recently described [18].

Transfection of IEC6 Cells

IEC-6 (ATCC, USA) cells were transfected with 300 ng of NF-κB-Luc reporter plasmid (20) and 10 ng of Renilla-Luc control plasmid (pRL-CMV, Promega, Madison, WI, USA) using Lipofectamine 2000 reagent (Invitrogen, Waltham, Massachusetts, USA) according to manufacturer's instructions. Subsequently, the cells were incubated for 18 hours with glucans (0.5 mg/mL) and then cells were incubated for an additional 6 h with or without TNF-α (0.05 μg/mL). Luciferase assay was performed using Promega dual luciferase assay kit (Promega, USA) according to manufacturer's instructions; luminescence quantification was performed using a TriStar LB941 microplate reader (Berthold technologies). The ratio of firefly/renilla readings for each sample was measured. The effect of the treatment relative to the not treated group is expressed as fold luciferase activity, determined by dividing the ratio of the firefly/renilla readings in each experimental sample by the average ratio of the firefly/renilla readings in the control group, as described below. Three independent experiments were performed in triplicate.

Luciferase activity (fold) = ratio of firefly/renilla luciferase reading (each sample) / average of the ratio of firefly/renilla reading (Control)

Nitric Oxide Production

Nitric oxide was determined by measuring the amount of nitrite, a stable end-product of NO, in the cell culture supernatant using a nitrate/nitrite colorimetric assay kit (Cayman chemicals; USA item no. 780001). Briefly, the murine macrophage cell line J774A.1 were seeded ($5x10^5$/500μl) on a 24-well plate, treated with or without glucans (200 μg/ml) for 2 hours, and then stimulated with LPS (1μg/ml) for 24 hours. Supernatants (80 μl) were collected and added to each well. To convert nitrate to nitrite 20 μl mixed solutions of nitrate reductase cofactor and nitrate reductase enzyme was added to each well and incubated for 2 hours in room temperature. Next, 50 μl of Griess reagent 1 (sulphanilamide) and 50 μl of Griess reagents 2 (N-(1-naphthyl) ethylenediamine) were added to each well and incubated at room temperature for ten minutes. Absorbance was measured at 540 nm using a microplate reader (synergy 2, BioTek Instruments, USA). The concentration of NO was determined by using nitrate + nitrite standard curve.

RNA Extraction and Real Time PCR

For all *in-vitro* RNA was extracted using Trizol and Pure link mini kit (Invitrogen, USA) according to manufacturer instructions. RNA extraction from colon of DSS treated animals was extracted

as described above and then subjected to further purification using LiCL as described by Viennois et al [21]. Real time assays were performed using ABI 7300 PCR and quantified to GAPDH gene by the ABI software (USA). All primers are listed in Table 1

Table 1: Mouse primers used in the real-time PCR experiments

Primer name	Forward	Reverse
TNF-α	GTCTGTGCCTCAGCCTCTTC	GCTTGGTGGTTTGCTACGAC
IFN-γ	5' CTGGACCTGTGGGTTGTTGAC 3'	5' CTGGACCTGTGGGTTGTTGAC 3'
IL-1β	5' CATCAGAGGCAAGGAGGAAAAC 3'	5' CATCAGAGGCAAGGAGGAAAAC 3'
IL-6	5' TTGCCATTGCACAACTCTTTTC 3'	5' TTGCCATTGCACAACTCTTTTC 3'
IL-12	5' GAAGCTGGTGCTGTAGTTCTCATATTT 3'	5' GAAGCTGGTGCTGTAGTTCTCATATTT 3'
GAPDH	5' TCCCATTCTCGGCCTTGAC 3'	5' TCCCATTCTCGGCCTTGAC 3'

In Vivo Examinations

Animal care and experimental procedures were in accordance with the guidelines of the animal ethics committee of the Hebrew University of Jerusalem. A total of 65 C57BL/6 mice were used for the experiment. Mice were divided into 12 groups of 5-7 mice in each group. Three groups for each glucan treatment two were given DSS and one served as control. Negative control received no treatment (glucans or DSS) and positive control consisted of two groups receiving DSS only. After 4 days of acclimation mice were fed per-os 50mg/mice per day glucan extracts from *P. eryngii* grown on either 80% OSMW, 20% OSMW or commercial α-glucan (Pullulan, Megazyme, Ireland) for 7 days. After the 7th day DSS was supplemented to some of the treatment groups. DSS was provided as 3% (w/v) in the drinking water for 5 additional days. At the end of the five days of DSS-treatment mice were sacrificed.

Histology Damage Score and Colon Length

After sacrifice, the whole colon was removed and length was measured. The distal third of the colon was fixed in 4% paraformaldehyde solution. Histological damage scoring was calculated on paraffin-embedded haematoxylin stained sections according to Erben et, al [22]. Randomized pictures were scored on a double blind assay by two individuals that were not related to the experiment. Score was given on a scale of 0-6. Where 0 (none), 3 (transmural) and 6 (severe entire crypt and epithelium lost). Each individual scored each image 3 times. Final grading is based on an average of 6 scores.

Measurement of cytokines (IL-1β and INF-γ) and chemokines

Blood was collected from the orbital vein and plasma separated using centrifugation 14,000 rpm, 10 mins, 4°C. 5 mm colonic samples were removed and placed in DMEM medium (D5796 Sigma Aldrich) over-night at 37°C and 5% CO2. Both the plasma and the medium were used for initial large scale cytokine screen covering various cytokines and chemokines (Mouse Cytokine Array G2, RayBiotech) according to manufacturer instructions. The incubation media containing samples of colonic fragments was additionally assayed for IL-1β and INF-γ using ELISA kit (RayBiotech, USA) according to manufacturer instructions.

Statistical Analysis

All analysis was performed using one-way ANOVA and comparison on means. Tulkey-Kramer was used for comparison of all groups and Dunnett's was used when all groups were compared to control. Results are presented as mean ± SEM. All figures show results from at least two independent experiments.

Myeloperoxidase Activity

The level of tissue myeloperoxidase (MPO) was determined by standard enzymatic procedure as described previously (Krawisz, Sharon, & Stenson, 1984) with some modifications [34]. Briefly, 1 cm of colonic tissue specimen was homogenized on ice in sodium phosphate buffer (pH 6•5) with 0•5% (w/v) hexadecyltrimethylammoniumbromide (HTAB) by three 30s pulses in a Polytron homogenizer. The homogenate was then sonicated for 20s following each of three 15 min freeze–thaw cycles. The sample was centrifuged at 15,000 g for 30 min at 4°C, and then stored at -80°C for subsequent measurement of MPO activity. The sample (100 ml) was added to 2•9ml phosphate buffer (pH 6•5) containing O-dianisidine hydrochloride (0•167 mg/ml) and 0•0005% (v/v) H2O2. Absorbance was measured at 460 nm using a spectrophotometer (GENESYS 10S Vis; Thermo Fisher Scientific) at 25 °C. One unit of MPO activity was defined as the value that can degrade 1 mmol H2O2 per min at 25°C. The values were then calculated as product concentration (U/ml).

Measurement of plasma cytokines and chemokines by the Mouse Cytokine Array G2, Ray Biotech

Blood was collected from the orbital vein and plasma separated using centrifugation 14,000 rpm, 10 mins, 4°C. The plasma was used for initial large scale cytokine screen covering several cytokines and chemokines (Mouse Cytokine Array G2, RayBiotech) according to manufacturer instructions.

The Cytokine array was scanned with a gene microarray laser scanner. Densitometry and analysis was performed according to the established Ray Biotech protocol.

Values of most of the cytokines values measured in the plasma of mice treated with DSS were upregulated except for IL-10, which is expected since it has anti-inflammatory effects and IL-9 which also has been shown to induce anti-inflammatory effects or alternatively induce resolution of inflammation.

Additionally, cytokines values measured in the plasma from mice treated with DSS together with glucans extracted from *P. eryngii* stalks grown on 80 % OMSW were closer to the range of Control mice.

Table 2: Analysis of glucan content under different growth conditions

	Growth substrate	Mean α-glucan		Mean β-glucan		Mean total glucan	
Pleurotus Eryngii	20% OSMW	20.18	±1.29	33.35	±4.91	56.18	±3.74
Pleurotus Eryngii	80% OSMW	18.3	±1.5	39.39	±3.42	57.7	±2.35

Measurements represent mean ± SEM of three samples

Results

In vitro

Measurement of Glucan Content in Glucan Extracts

Table 2 summarizes the glucan content (α-glucans, β-glucans and total glucans) in the different extracts from caps and stalks of P. eryngii grown on cultivation media including OMSW at different concentrations. Glucan content was determined by the Megazyme kit. α-Glucan was slightly higher on 20% OSMW supplemented substrate β-glucan was generally higher when *P. eryngii* was cultivated on substrate suplemented with 80% OSMW. The maximal concentration of percentage of total glucans was obtained in *P. eryngii* cultivated in 80% OMSW.

Attenuation of Nf-Kb in Response to Glucan Administration Following Stimulation With TNF-A

The transcription factor Nf-κB plays a pivotal role in inflammation and its over-expression and nuclear translocation is indicative of initiation of inflammation. IEC-6 cells derived from small intestine of rats were transiently transfected with a plasmid containing the Nf-κB enhancer elements (κB sites) fused to the luciferase gene [20]. 24 h after transfection glucan extracts harvested from P. eryngii stalks grown on either 20% or 80% OMSW and dissolved in ddH_2O as described in methods were added to the cell's culture media (25 mg/mL medium) for 18h after which TNF-α was added for additional 6 h of incubation. TNF-α is a known inducer of Nf-κB transcription [23]. Luciferase activity was indicative of activation of NF- κB (Figure 1b) (see methods). We then measured extent of transcription of the genes associated inflammation iNOS and TNF-α using Real Time PCR (see Figure 1a and 1c). Both glucan extracts reduced Nf-κB activation as measured by luciferase activity assay but the most effective treatment was exerted by glucans extracted from *P. eringii* stalks grown on 80% OMSW when compared to TNF-α without pretreatment (1.83±0.12 and 2.9±0.28 respectively) (See Figure 1b). Additionally, TNF-α gene expression was significantly reduced following pretreatment with both 80% and 20% OSMW (9.78 ± 3 and 9.36 ± 0.9 respectively) when compared to TNF-α without pretreatment (31.38 ± 4.3). iNOS is the gene in charge of nitric oxide production, which itself is a marker for inflammation. Interestingly, the expression of the iNOS gene was reduced by glucan extract from *P. eryngii* grown on 20% OSMW (1.34 ± 0.14 SEM) but not by glucans extracted from P. eryngii grown on 80% OSMW (4.8 ± 0.21 SEM) when compared to no TNF-α without pretreatment (6 ± 0.33 SEM) (Figure 1c).

Nitric Oxide Production in Lipopolysaccharide (LPS) Treated J774A.1 Macrophages

Macrophages are the first line of defense in an event of inflammation. In chronic bowel inflammation, macrophages are activated and as a result, they damage the intestinal tissue. Nitric oxide is a small free radical secreted by activated macrophages, it functions as an inflammatory mediator and in high amounts has cytotoxic capabilities. To assay the ameliorating properties of dissolved glucan extracts prepared from *P. eryngii* mushrooms grown on substrates containing different OSMW percentages the microbial-derived activating factor LPS was added in order to activate the J774A.1 murine macrophages. J774A.1 cells were seeded and pretreated with glucans from *P. eryngii* grown on either 20% or 80% OMSW for 2h and then stimulated with LPS for 24h. NO production was measured using Griess reaction. Both glucan preparations (from 20 % OMSW and 80 % OMSW) significantly reduced the production of NO from J774A.1 cells as compared to LPS stimulation alone i.e. non-glucan treated. Glucans from mushrooms grown on 80% OMSW exerted the reducing activity more significantly (16.5 μM ±1.5 SEM difference).

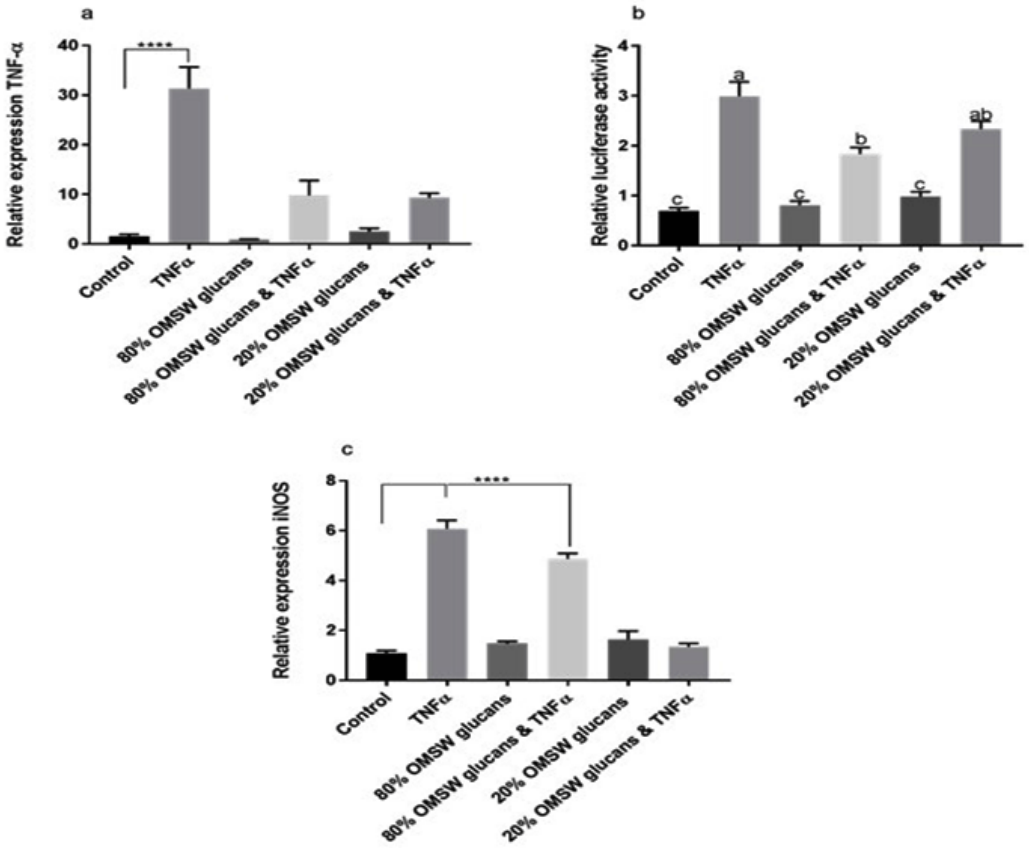

Figure 1: Nf-κB activation in response to glucan administration following stimulation with TNF-α
Effect of pre incubation of IEC-6 cells with glucans and assessment of Nf-κB induction followed by TNFα activation, effect on TNF-α, and iNOS transcripts. a; Real Time PCR of TNF-α gene expressed by IEC-6 cells followed by treatments. b; Luciferase activity in response to NF-κB activation by TNF-α. c; Real Time PCR of iNOS gene expressed by IEC-6 cells. Bars represent four biological repetitions and values represent mean ± SEM. In a and in c **** indicate significant difference compared to control ($P < 0.0001$) in b letters indicate statistical significance between all groups ($P < 0.0001$)

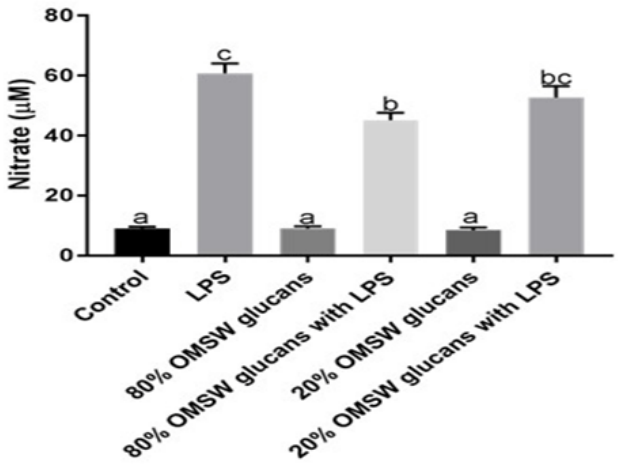

Figure 2: Nitric Oxide production in J774A.1 macrophages
Nitrate production (μM) was measured in J774A.1 macrophages pre-treated with glucan extracts (18h) and stimulated with LPS (2h). Bars represent four repetitions and values represent mean ± SEM. Letters represent significant difference between groups ($P < 0.0001$)

In vivo

Effects of Isolated Glucans on Acute Colitis Induced in Mice Treated With Dss

The in-vivo experimental design is depicted in Figure 3 and further elaborated in material and methods. In short, mice were divided into 8 major groups. Six groups were fed glucan extracts from P. eryngii grown on either 20% OSMW, 80% OSMW or commercial glucans (Pullulan) (50mg/mice/day) for 7 days. Control and DSS-treated groups were not fed with glucans. After seven days of dietary treatment, colitis was induced by DSS administration in the drinking water (3% w/v) in four groups for additional five days. At the end of this treatment schedule animals were sacrificed and tissues obtained for analysis. The mice groups fed with glucans received these mushroom preparations until the end of the experiment.

One of the most important determinants resulting from DSS-treatment in order to induce acute colitis mice is enhanced intestinal damage as evidenced by assessing histologic damage score of the large intestine. Figure 4 and Figure 5a clearly demonstrate that the histologic damage score is significantly

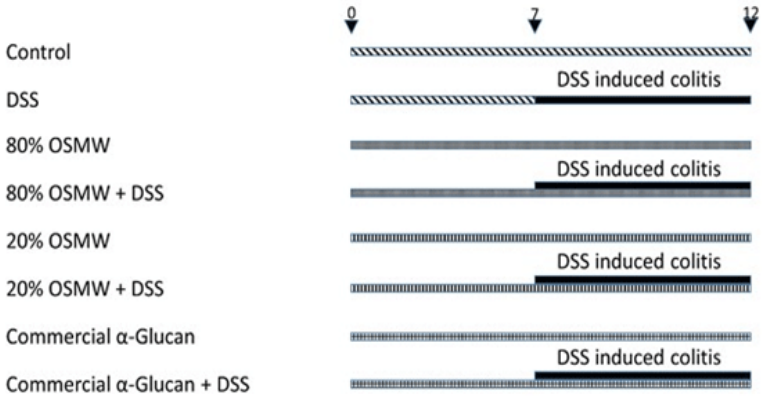

Figure 3: Experimental design
Control and only DSS treated groups were not fed glucans throughout the whole experiment. Mice were fed Glucans extracted from P. eryngii grown on 80% and 20% OSMW or commercial α-glucan (Pullulan) from the first day. After 7 days, DSS was administered in the drinking water (3% w/v) to induce colitis in following groups: DSS, 80% OSMW + DSS, 20% OSMW +DSS and commercial α-glucan + DSS

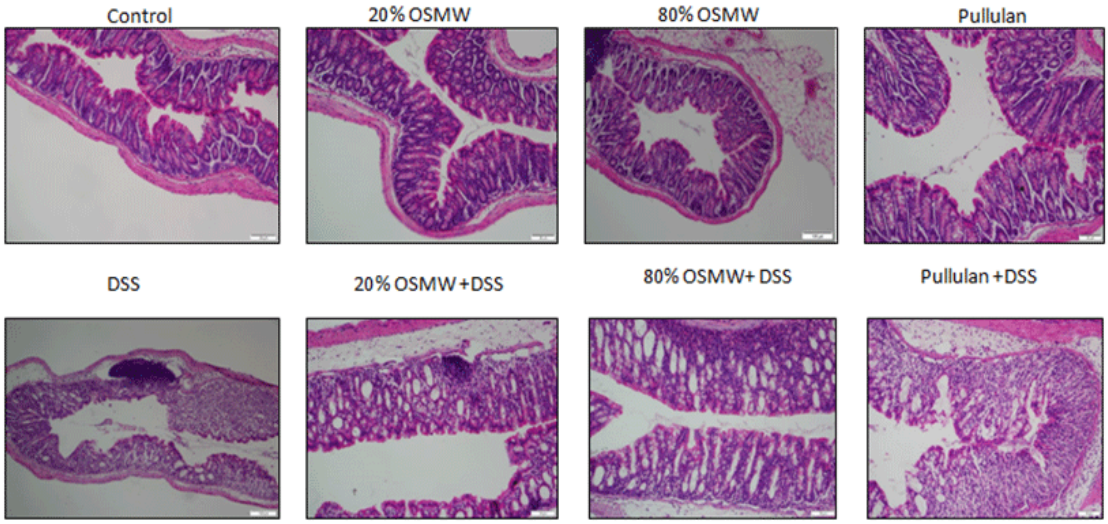

Figure 4: Histology of murine sections of the distal colon shown by haematoxylin and eosin staining (H&E)
Control mice; Mice fed glucans extracted from mushrooms grown on 20 % OMSW; Mice fed glucans extracted from mushrooms grown on 80 % OMSW; Mice fed Pullulan; dextran sodium sulfate (DSS)-treated mice; DSS-treated mice fed glucans extracted from mushrooms grown on 20 % OMSW; DSS-treated mice fed glucans extracted from mushrooms grown on 80 % OMSW; DSS-treated mice fed Pullulan

higher as a consequence of DSS treatment in C57BL/6 mice. Glucan extracts prepared from *P. eryngii* grown on 20% OSMW and commercial glucans significantly reduced histology damage compared to only DSS treatment alone. Colon shortening is also a known phenomenon resulting from DSS treatment during DSS-induced colitis (See Figure 5b). While treatment with the various glucan preparations did not completely recover the original loss in colon length compared to control, nonetheless, all glucan treatments attenuated colon shortening (Figure 5b).

Effects of Isolated Glucans Prepared From P. Eryngii Mushrooms Grown on Different OSMW Percentages of Growth Substrate on the Expression of Colonic Inflammatory Related Genes

Essentially, all glucan extracts reduced the colonic expression of several genes encoding interleukins and chemokines. Glucans prepared from *P. eryngii* mushrooms grown on 20% OSMW showed a stronger reduction in gene expression of

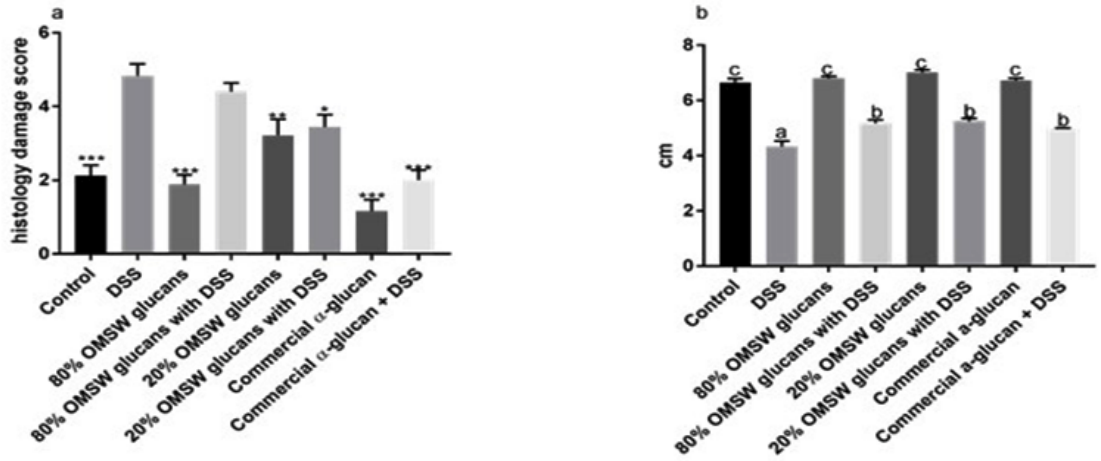

Figure 5: Histology damage score in colonic samples of DSS-treated C57BL/6 mice
A; DSS-treated C57BL/6 mice fed with glucans prepared from P. eryngii grown on 20% OSMW and commercial α-glucan significantly reduced histology damage compared to only DSS treatment. B; All orally administrated glucans attenuated colon shortening induced by DSS treatment. Bars represent N = 5 in non-DSS-treated groups and N = 10 in DSS-treated groups. Values represent mean ± SEM. A; asterisks represent significant difference from control, * P < 0.0153, ** P < 0.003; *** P< 0.0001 B; Different letters represent significant difference between all groups P < 0.0001

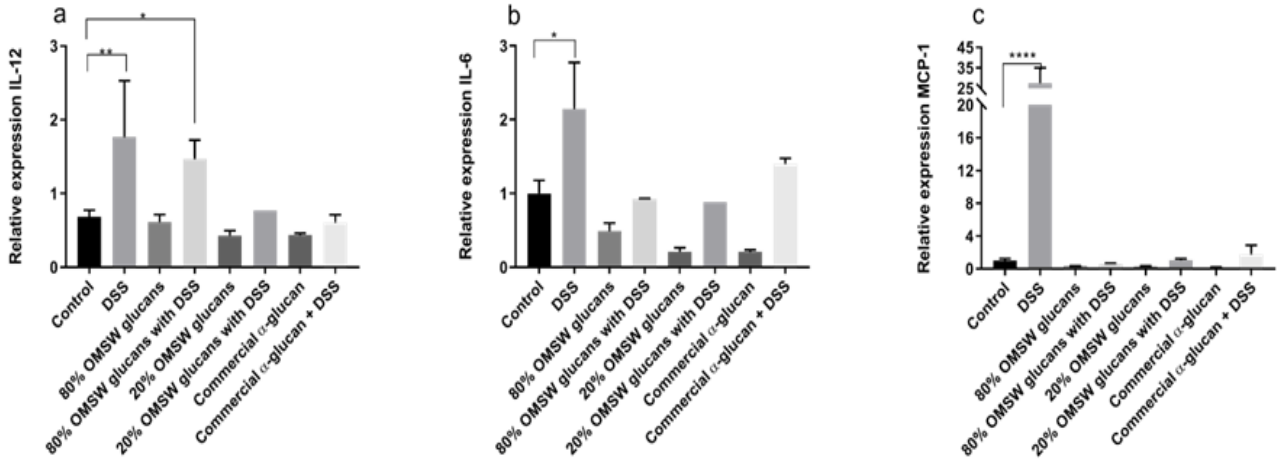

Figure 6: Gene expression of inflammation related genes in mice colonic samples as quantified by Real Time PCR
a; IL-12 relative expression, b; IL-6 relative expression, c; MCP-1 relative expression,. Glucans were administered per os 7 days prior to DSS treatment. N=5 bars represent mean ± SEM. Statistical analysis in all figures compared treatments to control. a * *P* < 0.0360; ** *P* < 0.086, b *P* <0.0205, c *P* <0.0001

interleukins and chemokines than glucans prepared from *P. eryngii* mushrooms grown on 80% OSMW. For example, for IL-12, treatment of mice with glucans from *P. eryngii* mushrooms grown on 80% OSMW mushrooms reduced expression of the colonic gene when compared to DSS treated mice however the effect was significantly stronger when mice were treated with glucans prepared from *P. eryngii* mushrooms stalks grown on 20% OSMW. IL-12 expression was not significantly different from control in mice treated with commercial glucans (Figure 6a). The same pattern was observed for the colonic gene transcript IL-6 (Figure 6b). Monocyte chemo attractant protein (MCP)-1 is an important chemokine in the pathology of IBD in both model animals and humans [24]. Glucans dramatically reduced its gene expression (Figure 6c). Myeloperoxidase activity is a marker for acute inflammation in the gut [6]. Myeloperoxidase activity was measured as described previously and provided in supplemental methods [1, 25]. Glucans extracts reduced Myeloperoxidase activity yet the reduction did not reach statistical significance (See Figure 7).

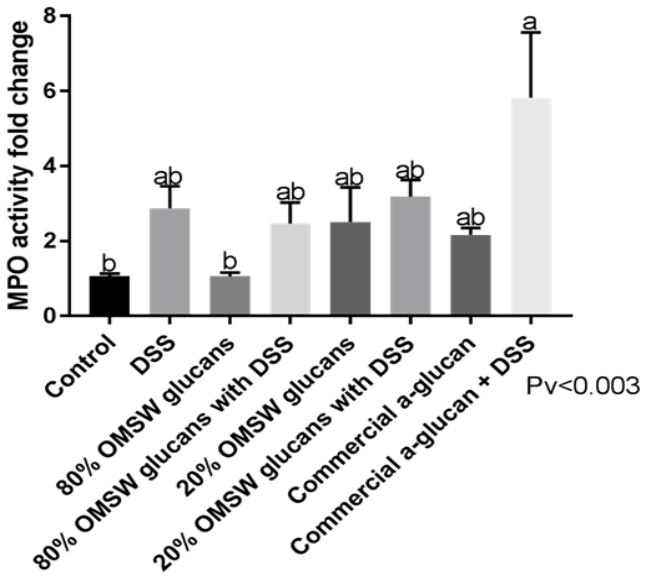

Figure 7: Myeloperoxidase activity is mice colon. Activity depicted in fold change to control. Treatment with DSS or glucans increased MPO activity. Bars represent mean and SEM, n=5

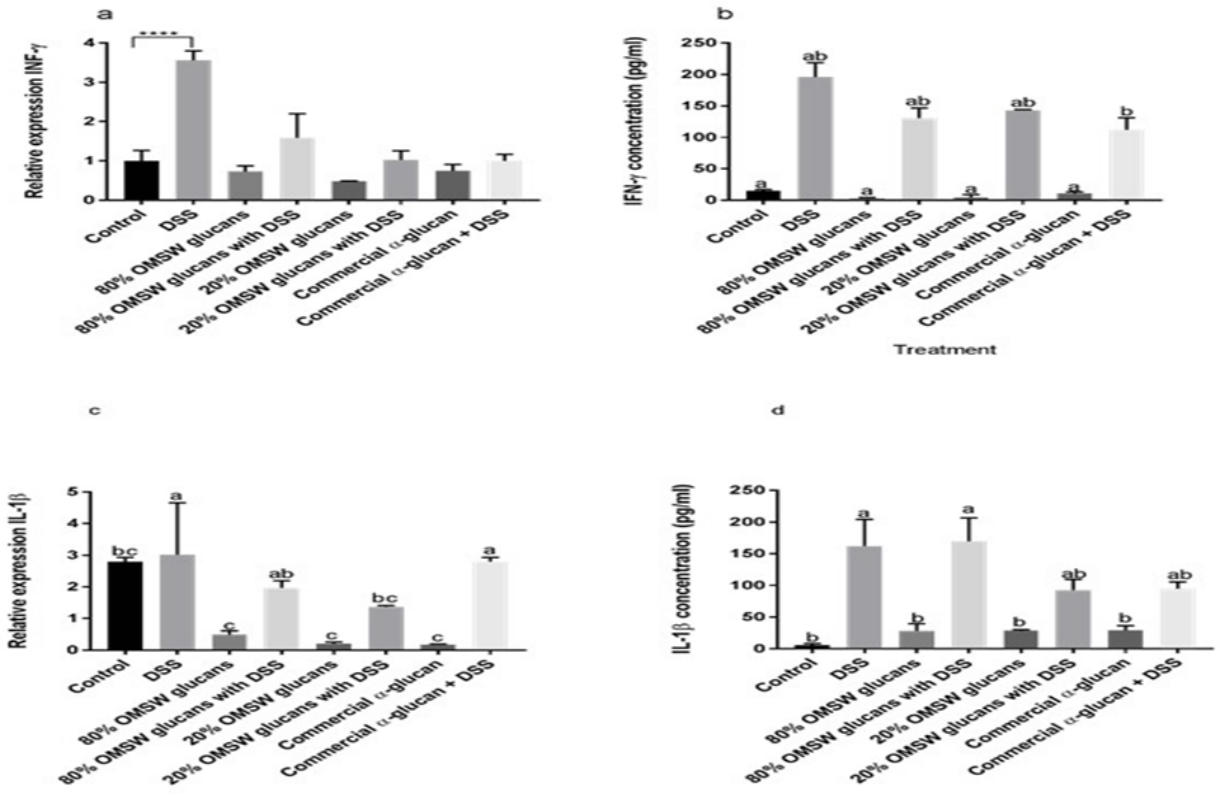

Figure 8: Quantification of gene expression and chemokines secretion from colonic samples
Relative gene expression in the colon of INF-γ and IL1-β as quantified by Real time PCR, is shown in a and c. Chemokine secretion quantified by ELISA is shown in b and d. Bars represent mean ± SEM; N=5. B-c significance was calculated between all groups except in a where all groups were compared to control. a and b $P < 0.0001$ c. $P < 0.001$, d $P < 0.007$

Effects of Isolated Glucans on Expression and Secretion of INF-Γ and IL1-B in Colonic Segments

Both INF-γ and IL1-β are secreted during active colitis in humans and DSS induced colitis in mice from colonic tissue. INF-γ is in fact an essential factor for induction of colitis [26, 27]. IL1-β is secreted mainly by the innate immune cells in response to inflammatory trigger [28]. 5mm colonic fragments were incubated overnight in DMEM medium.

The harvested medium was removed and initially assayed for several cytokines measured by the Mouse Cytokine Array G2, Ray Biotech (Table 3). These initial results led us to focus on INF-γ and IL1-β using ELISA (see methods). The secreted chemokines were compared to extent of gene expression in the colon. Both the gene expression and secretion of IL-1β and INF-γ were significantly increased by DSS induced colitis (Figure 8). Expression of INF-γ was reduced by pretreatment with glucan extracts to the same levels of control yet chemokines secreted in the colon showed less reduction (Figure 8a & 8b). In IL1-β the expression and secretion profile were similar. Pretreatment with glucan extracts results in reduced expression and secretion of both INF-γ and IL1-β.

Additionally, plasma cytokine levels were also analyzed for various cytokines and the results are summarized in Table 3. These results essentially indicate that DSS treatment affects dramatically the plasma cytokine profile of mice as compared to control and most of all cytokines and chemokines evaluated are up regulated. Mice treated with glucans prepared from *P. eryngii* mushrooms stalks grown on 80% OSMW and DSS have diminished plasma cytokine levels as compared to DSS. Additionally, it is evident that mice treated only with glucans prepared from *P. eryngii* mushrooms stalks grown on 80% OSMW show some priming of the immune response. The results are only indicative since the values obtained results from densitometry assessments.

Table 3: Plasma cytokines and chemokines levels measured by the Mouse Cytokine Array G2, RayBiotech

Cytokine	Control densitometry	DSS densitometry	80% OMSW densitometry	80% OMSW+DSS densitometry
CTACK	774	4,858	1,158	1,373
GCSF	563	713	676	509
GM-CSF	444	33,135	3,096	13,126
IL-2	435	782	686	681
IL-3	442	899	620	761
IL-4	467	740	619	561
IL-5	507	889	714	715
IL-6	575	771	692	717
IL-9	772	777	760	717
IL-10	899	712	1,329	1,185
IL-12 p40p70	661	1,229	1,115	755
IL-12p70	652	712	535	604
IL-13	411	774	588	612
IL-17	453	1,006	814	760
IFN-γ	498	596	489	514
KC	319	482	511	590
Leptin	348	1,029	925	493
MCP-1	717	941	790	800
MCP-5	677	635	426	541
MIP-1a	383	827	760	696
MIP-2	539	639	446	574
MIP-3 b	467	1,148	827	661
RANTES	496	709	750	675
SCF	450	8,638	3,454	3,880
TARC	575	1,085	770	924
TNF-α	535	851	790	733
Thrombopoietin	670	744	638	671

Discussion

The effect of glucans on reduction of inflammation has been abundantly documented earlier [5, 6, 9-11]. In previous studies, we have shown that supplementing the P. eryngii growth substrate with Oil Mill Solid Waste (OMSW) resulted in induced increased glucan content and that glucans accumulate mostly in the stalks of the mushroom [8, 18]. In this study, we aimed at investigating the biological effect exerted by glucans extracted for stalks of *P. eryngii* grown on either 20% or 80% OSMW in vitro, on inflammation induced in epithelial cells and in macrophages and in-vivo in acute intestinal inflammation induced by DSS treatment. We firstly measured α-glucan, β-glucan and total glucan in extracts from P. eryngii grown on 20% and 80% OSMW (Table 2). The results indicate, as previously reported that when the growing soil is enriched with 80% OSMW the resulting P. eryngii grown under these conditions produce significantly more glucans [18].

We then measured the effect of the extracted glucan samples on the expression of Nf-κB in rat epithelial cells (IEC-6). Nf-κB mediates a plethora of cellular immune-regulatory responses [15, 23]. Cells were pretreated with glucans before the induction with TNF-α. As expected, TNF-α dramatically upregulated the expression of luciferase located upstream to Nf-κB enhancer element (Figure 1b). IEC-6 transfected cells pre-treated with glucans extracted from P. eryngii grown on 80% OSMW significantly reduced activation of Nf-κB. Additionally, TNF-α gene expression was down regulated by pretreatment with both glucans extracted from *P. eryngii* stalks grown on substrate containing 80% and 20% OSMW.

Nitric Oxide production is a characteristic event following pathogen attack and concomitant inflammation. Nitric Oxide expression is activated via the Nf-κB pathway [29]. Indeed, TNF-α triggered more than 6-fold the increase in expression of the iNOS gene. Pre-incubation with glucan extracts significantly reduced the expression iNOS only with glucans extracted from P. eryngii grown on 20% OSMW. This emphasizes the complexity of the effects of glucans on the inflammatory process and the resulting differential genetic response to stimulation/inhibition mediated by glucans.

During active colitis or other IBD stages, the intestinal activated macrophages induce severe injury of the intestinal epithelium. Nitric oxide is a characteristic event of activated macrophages. We simulated in vitro this event when we treated macrophages with LPS in vitro and measured induced NO production increase by ~6 fold as a result of LPS treatment. Glucans reduced LPS induced NO significantly; however glucans harvested from *P. eryngii* grown on 80% OSMW exhibited a stronger inhibition of NO production compared to glucans from *P. eryngii* grown on 20% OSMW (16.5 μM ± 1.5 and 8 μM ± 3.8 respectively, see Figure 2). Our findings in conjunction with other reports showing inhibition of NO production in LPS-activated macrophages incubated with glucans demonstrate the important immune-modulating effects of glucans, especially those harvested from P. eryngii edible mushrooms [9, 14, 30, 31].

We recently conducted an *in-vivo* study in which we induced IBD in BALB/c mice via treatment with DSS [8]. This study demonstrated that the expression of the inflammation gene markers MIP-2, TNF-α, INF-γ, CXCL1 and iNOS were significantly reduced in mice treated with glucan extracts, additionally we showed that the number of activated monocytes were significantly reduced in glucan treated mice [8]. DSS induces severe colitis in mice model, however the extent and characterization of the inflammation differs between mice strains [32]. BALB/c mice show recovery in body weight, inflammatory score, diarrhea score, colonic damage and inflammatory cytokines short after cessation of DSS administration [33]. Under the same conditions C57BL/6 develop chronic inflammation subsequent to the acute response with cytokine levels remaining high even after cessation of DSS administration [33]. Therefore, comparison of the results of our previous study conducted in Balb/c mice to the results of the present study conducted in C57BL/6 allows to appreciate the effects of glucans in mice suffering from DSS-induced IBD-inflammation at different degrees [8].

In the present study, glucans treatment shows some protective properties on the colon length and the histological damage score on DSS induced colitis (Figure 4 & 5). Nevertheless, glucan pretreatment did not induce the recovery of colon lengths to the levels of untreated control (Figure 5b). Our data indicate that the expression of inflammatory cytokines MIP-2, TNF-α, IL-12 and IL-6 were not reduced in glucan treatments compared to control (data not shown); we demonstrate that INF-γ and IL-1β protein levels in intestinal samples were reduced yet not significantly in most of the treatments (Figure 8). Changes in gene expression as measured by q-PCR were more robust for INF-γ expression and less for IL-1β.

Cumulatively, the results of the present study demonstrate that pretreatment of C57BL/6 mice with glucan extracts from *P. eryngii* cultivated on either 20 % OMSW or 80 % OMSW reduces inflammation in DSS induced acute colitis. Due to the severity of inflammation as a result of DSS treatment of C57BL/6 mice our results were not as conclusive as our previous studies performed by similar glucan extracts on BALB/c mice emphasizing the importance of stage of inflammation when treating with glucans [8].

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