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1 **Effects of α -glucans from *Agaricus bisporus* on *ex vivo***
2 **cytokine production by LPS and PHA stimulated PBMCs; a**
3 **placebo-controlled study in slightly hypercholesterolemic**
4 **subjects**

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7

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22 **Abstract**

23 Mushrooms are known for their immune modulating effect for which the
24 polysaccharide fraction, mainly glucans, seems to be responsible. Fungal β -
25 glucans have been studied extensively, whereas little is known about
26 mushroom α -glucans. We have earlier shown that the polysaccharide fraction
27 from the mushroom *A. bisporus*, consisting for 90% of α -glucans, induced *in*
28 *vitro* tumor necrosis factor (TNF) α and nitric oxide production. The purpose of
29 the present study was to evaluate the effects of consuming *A. bisporus* α -
30 glucan on *ex vivo* cytokine production by human peripheral mononuclear
31 blood cells (PBMCs).

32 A double-blind randomized trial was designed in which fifty-six mildly
33 hypercholesterolemic subjects consumed a control fruit juice with no added α -
34 glucans (200 mL/day) for a 2 weeks run-in period. For the next 5 weeks, the
35 control group (N=30) continued consumption of the control fruit juice, while the
36 intervention group (N=26) consumed the same fruit juice enriched with α -
37 glucans from *A. bisporus* (5 g glucans/day). Changes in interleukin (IL)-1 β , IL-
38 6 and TNF α cytokine production by lipopolysaccharide (LPS)-stimulated
39 PBMCs were evaluated, as well as changes in T-helper (Th)1/Th2 cytokines
40 by phytohemagglutinin (PHA)-stimulated PBMCs.

41 Consumption of *A. bisporus* α -glucans lower LPS-induced TNF α
42 production by 69% (p=0.017) as compared to the control group, whereas no
43 effect on IL-1 β and IL-6 was observed. No obvious Th1-Th2 skewing by PHA-
44 stimulated PBMCs was observed. However, we observed a trend towards a
45 decreased production of IL-12 and IL-10.

46 Our current finding suggests that *in vivo*, α -glucans have lost their
47 efficacy to stimulate the immune response as observed **in our *in vitro* mouse**
48 **model.**

49

50

51 Abbreviations used: IFN, interferon; IL, interleukin; LPS, lipopolysaccharide;
52 PBMC, peripheral mononuclear blood cell; PHA, phytohemagglutinin; TNF,
53 tumor necrosis factor; Th, T-helper

54

55 Running title: Immune modulating effect of *A. bisporus* α -glucans

56

57 Keywords: *Agaricus bisporus*, α -glucan, cytokines, immune modulation,
58 nutrition, peripheral mononuclear blood cell

59

60 **Introduction**

61 Mushrooms have been studied extensively for their immune modulating and
62 anti-tumor properties (Brown *et al.*, 2003; Borchers *et al.*, 2004; Lull *et al.*,
63 2005; Chen and Seviour, 2007; Wei *et al.*, 2008). Indeed, there are many
64 potentially biologically active compounds present in mushrooms, e.g. small
65 proteins, lectins, polyphenols and polysaccharides. It is assumed that the
66 polysaccharides are a main bioactive part of the mushrooms, at least in
67 relation to their immune modulating effects (Wasser, 2002). These
68 polysaccharides are mainly β -glucans that are present in the cell walls. In
69 addition, also α -glucans are present in the polysaccharide fraction of
70 mushrooms (Zhang and Cheung, 2002). Whereas the effects of β -glucans
71 have been studied extensively, little is known about the immune modulating
72 effect of mushroom α -glucans. We have reported earlier that a polysaccharide
73 fraction from *Agaricus bisporus*, which contained 90% α -glucan increased
74 nitric oxide and tumor necrosis factor (TNF) α production by bone marrow-
75 derived macrophages from mice *in vitro* (Volman *et al.*, in press).
76 Furthermore, Bittencourt *et al.* (Bittencourt *et al.*, 2006) have reported that α -
77 glucans from *Pseudallescheria boydii* stimulated the *in vitro* secretion of TNF α
78 and interleukin (IL)-12 by mice macrophages and dendritic cells. This
79 increased secretion of IL-12 indicates a polarization of naïve T-cells into T-
80 helper (Th) type 1 skewed responses, which is generally attributed to
81 mushroom β -glucans (Suzuki *et al.*, 2001; Saito *et al.*, 2003; Lin *et al.*, 2006;
82 Takimoto *et al.*, 2008). In addition, animal studies have also shown that
83 consumption of *A. bisporus* stimulated the immune response (Wu *et al.*, 2007;

84 Ren *et al.*, 2008), but the bioactive fraction from these extracts is not known. It
85 is possible that these immune-modulating effects were due to *A. bisporus*
86 derived α -glucans extracts, which have been studied to some extent *in vitro*
87 and in animals. Effects of consuming these extracts on *ex vivo* cytokine
88 production by isolated and stimulated human peripheral mononuclear blood
89 cells (PBMCs) have however, to the best of our knowledge, not been studied
90 in well-controlled human trials. Therefore, the purpose of the present study
91 was to evaluate PBMC cytokine secretion patterns after consumption of α -
92 glucan rich fractions from *A. bisporus* incorporated into fruit drinks in humans.
93 For this, PBMCs were isolated and stimulated *ex vivo* by two approaches.
94 First, we measured IL-1 β , IL-6 and TNF α production after lipopolysaccharide
95 (LPS) stimulation and secondly we evaluated a potential shift in the Th1/Th2
96 balance of secreted cytokines (IL-2, IL-4, IL-5, IL-12, IL-13, IL-17 and
97 interferon (IFN)- γ) after phytohemagglutinin (PHA) induced T-cell proliferation
98 of isolated PBMCs.
99

100 **Methods**

101 **Subjects**

102 Fifty-six apparently healthy men and women with slightly elevated serum total
103 cholesterol concentrations (between 6.0 – 8.0 mmol/L) were included in this
104 study. Volunteers were recruited from Maastricht and surrounding villages by
105 advertisements in local newspapers. In addition, subjects who had
106 participated in earlier studies at our department were approached. Inclusion
107 criteria were stable body weight (weight gain or loss <3 kg in the past 3
108 months); Quetelet index <32 kg/m²; systolic blood pressure <160 mmHg, and
109 diastolic blood pressure <95 mmHg; no presence of glucosuria, proteinuria or
110 anemia; no use of medication or a prescribed diet known to affect lipid or
111 glucose metabolism; no history of coronary heart disease, cancer, diabetes,
112 kidney, liver, pancreatic disease, or malignancies <5 y ago; no abuse of drugs
113 and/or alcohol; and no pregnant or breast-feeding women, serum
114 triacylglycerol concentrations <3.0 mmol/L and serum total cholesterol
115 concentrations between 6.0 and 8.0 mmol/L. Baseline characteristics are
116 shown in table 1. There were no statistical differences between the two
117 groups in these baseline characteristics. The subjects were requested not to
118 change their habitual diets, levels of physical exercise, smoking habits, or use
119 of alcohol during the study. Blood donation or participation in another
120 biomedical trial was not allowed 30 d before the start and during the study.
121 The Medical Ethical Committee of the University of Maastricht approved the
122 study and all subjects gave written informed consent before entering the
123 study. Eight subject dropped out during the study - all during the run-in period

124 - because of various reasons not related to the experimental products, e.g.
125 holidays, difficulties during venipunctures.

126

127 **Study design**

128 The study had a placebo-controlled, double-blind, parallel design. Before the
129 study started, all 56 subjects were randomly divided into two groups. During
130 the first two weeks of the study (run-in period), all subjects consumed a fruit
131 juice (200 mL/day) without added α -glucans from *A. bisporus*. The 200 mL
132 was consumed at two occasions divided over the day, i.e. \pm 100 mL around
133 lunch and \pm 100 mL around diner. During the following 5 weeks intervention
134 period, the control group (N=30) continued consumption of the control fruit
135 juice, while the intervention group (N=26; α -glucan group) consumed the
136 same fruit juice but now enriched with α -glucans from *A. bisporus* (*A. bisporus*
137 juice providing 5 g glucans/day). The glucan extracts were especially for this
138 study prepared by Innerlife (Venlo, the Netherlands) and the procedure is
139 described by Volman *et al.* (Volman *et al.*, 2009). Briefly, semi-purified
140 polysaccharides were obtained from fruiting body material by hot water
141 extraction followed by ethanol precipitation as described before (Wei *et al.*,
142 2008). A final solution was made in phosphate buffered saline at 900 μ g/mL,
143 which was then heated for 1h at 100 °C and stored at 4 °C. α -(1 \rightarrow 4) Glucan
144 was obtained from *Agaricus bisporus* stipes by passage of the polysaccharide
145 extract over a DEAE-cellulose column. The colorless non-binding
146 polysaccharide has a MW > 200 kDa, which can be completely degraded by
147 α -amylase, and is characterized as an α -glucan. We later discovered that the

148 extract does not bind to a C18 column and runs as a single peak giving 100%
149 recovery (unpublished results). The polysaccharide fraction of the *Agaricus*
150 *bisporus* extract consist for 90% of α -(1→4) glucan. In addition approximately
151 10% of the polysaccharides complexes with polyphenolic compound and elute
152 from a DEAE cellulose column at 0.25-0.40 M NaCl. The general composition
153 of hot water mushrooms extract was published earlier (Wei and Van
154 Griensven, 2008) and showed a presence of approximately 5% (W/W)
155 polyphenols in the mixtures, whereas their protein content was usually less
156 than 2%. Therefore, the α -glucan extract could be considered as “highly
157 purified”. The α -glucan extracts were subsequently incorporated into the fruit
158 juices. The fruit juice was a blend containing ten different fruits and contained
159 per 100 mL 47.2 kcal, 11.3 g carbohydrate, 0.01 g fat and 0.47 g protein.
160 During the study, subjects recorded in diaries about their illnesses, medication
161 used, alcohol consumption, any deviations to the study protocol, and any side
162 effects experienced.

163 At the end of the run-in period (week 2) and the end of the intervention
164 period (week 7) blood was sampled after an overnight fast of at least 10
165 hours. Subjects were not allowed to consume alcohol 24h before blood
166 sampling and were not allowed to smoke the morning before blood sampling.
167 There were two smokers in both the control and the α -glucan group. Blood
168 was sampled in endotoxin-free heparinized tubes and serum tubes (Becton
169 Dickinson Vacutainer Systems, Franklin Lakes, NJ) for PBMC isolation and
170 clinical chemistry, respectively.

171

172 **PBMC stimulation**

173 To examine *ex vivo* cytokine production by PBMCs, cells were isolated from
174 16 mL heparinized whole blood by means of gradient centrifugation under
175 sterile conditions using Lymphoprep according to the manufacturers'
176 instructions (Nycomed Ltd, Birmingham, United Kingdom). After isolation,
177 PBMCs were immediately plated in 24-well flat-bottom culture plates.

178 For evaluation of IL-1 β , IL-6 and TNF α production, cells were diluted to
179 2.5×10^6 cell/mL and seeded. Cells were mixed with LPS (E Coli 055:B5,
180 Sigma, St Louis, MO) or polymixin B (Sigma) to a final concentration of 10
181 ng/mL. Polymixin B was used as a negative control as it binds to LPS. Cells
182 were cultured in RPMI-1640 with HEPES and L-glutamine (Invitrogen, Breda,
183 The Netherlands) supplemented with 1% heat-inactivated human serum pool,
184 1% penicillin streptomycin (Invitrogen) and 1% sodium pyruvate (Invitrogen).
185 The cells were incubated for 4 hours at 37 °C in a 5% CO₂ humidified
186 atmosphere. After incubation, the culture media were aspirated and
187 centrifuged at 1,500 RPM for 5 min to obtain cell-free media, which were
188 stored at -80 °C until further analysis.

189 For analysis of Th1/Th2 cytokine production, PBMCs were diluted to
190 1×10^6 cells/mL and seeded in 24-well flat-bottom culture plates. Next, 50
191 μ g/mL PHA (Roche, Mannheim, Germany) was added to initiate proliferation
192 of the CD4⁺ T cells. Medium was aspirated after 52h proliferation and
193 centrifuged at 14,000 RPM for 2 min. Culture media were stored at -80 °C
194 until further analysis for cytokines.

195

196 **Cytokine analysis**

197 IL-1 β , IL-6 and TNF α concentrations in supernatant of 4h stimulated PBMC
198 were assessed by commercially available MSD multiplex ELISAs (MSD,
199 Gaithersburgh, MD), as described by the manufacturers. Also IL-2, IL-4, IL-5,
200 IL-12, IL-13, IL-17 and IFN- γ concentrations were analyzed in supernatant of
201 52h stimulated PBMC, by custom panel MSD multiplex ELISAs (MSD).

202

203 **Clinical chemistry**

204 Serum samples collected at the end of the run-in and intervention period were
205 analyzed to determine parameters reflecting kidney and liver function
206 (creatinine, alanine transaminase, aspartate aminotransferase, γ -glutamyl
207 transpeptidase, alkaline phosphatase, and total bilirubin) by routine
208 procedures.

209

210 **Statistics**

211 Normality was tested by the Shapiro-Wilk test. Baseline characteristics are
212 presented as means \pm SD and were analyzed for statistical differences by an
213 independent-samples t-test. The baseline cytokine concentrations were tested
214 by the non-parametric Mann-Whitney test, as these were not normally
215 distributed. For each subject, responses in amounts of cytokines produced
216 were calculated as the change between the concentration at the end of the
217 run-in period (week 2) and those at the end of the intervention period (week
218 7). The same was done for clinical chemistry parameters. Cytokine
219 concentrations and clinical chemistry parameters are presented as medians

220 with ranges. Since the changes in concentrations of the cytokines, liver and
221 kidney function parameters were not normally distributed, the differences in
222 changes were tested by the non-parametric Mann-Whitney test. Differences
223 were considered statistically different at $p < 0.05$. All statistical analyses were
224 performed using SPSS 15.0 (SPSS, Chicago, IL, USA).

225

226 **Results**

227

228 **Effects of α -glucans from *A. bisporus* on cytokine production by LPS** 229 **stimulated PBMCs**

230 Concentrations of the cytokines IL-1 β , IL-6 and TNF α produced by LPS
231 stimulated PBMCs both at the end of the run-in period as well as at the end of
232 the experimental period were analyzed (table 2). At baseline there were no
233 differences between both groups. The median change in TNF α production by
234 PBMCs was 69% lower in the *A. bisporus* group (249 pg/mL, range -656 to
235 2025 pg/mL) as compared to the change in the control group (792 pg/mL,
236 range -1071 to 1675 pg/mL; p=0.017). **Individual changes in TNF α production**
237 **by PBMC are shown in figure 1.** No statistical differences were found for the
238 changes in IL-1 β and IL-6 between both groups. All polymixin B controls were
239 negative (data not shown).

240

241 **Effects of α -glucan rich fractions from *A. bisporus* on cytokine** 242 **production by PHA stimulated PBMCs**

243 The effects of the consuming α -glucan enriched drinks on cytokine production
244 by PHA stimulated PBMCs as compared to the effects of PBMCs isolated
245 from the control group are shown in table 3. At baseline there were no
246 differences between both groups. The change in IL-12 production after the
247 intervention period showed a trend towards a decreased production after *A.*
248 *bisporus* consumption (-10 pg/mL, range -79 to 37 pg/mL) as compared to

249 control drinks (4 pg/mL, range -51 to 53 pg/mL; p=0.059). Also the production
250 of IL-10 showed a trend towards a lower production after *A. bisporus*
251 consumption (-159 pg/mL, range -1454 to 918 pg/mL) as compared to the
252 control group (56 pg/mL, range -644 to 1343 pg/mL; p=0.053). There **was** no
253 effect of the α -glucan fraction from *A. bisporus* on the production of IFN γ , IL-2,
254 IL-13, IL-4 and IL-17 of PHA stimulated PBMCs (p-values for diet effects:
255 IFN γ , p=0.451; IL-2, p=0.274; IL-13, p=0.961; IL-4, p=0.791 and IL-17,
256 p=0.173) (table 3).

257 **Effects of α -glucan enriched drinks on kidney and liver function** 258 **parameters**

259 Parameters reflecting kidney and liver function were determined in serum
260 samples from all subjects at end of the run-in period as well as at the end of
261 the intervention period. Median creatinine, alanine transaminase, aspartate
262 aminotransferase, γ -glutamyl transpeptidase, alkaline phosphatase, and total
263 bilirubin concentrations were all within normal ranges at both occasions (table
264 4). Moreover, the changes were not different between the intervention and
265 control groups, except for creatinine, which showed a trend towards a lower
266 concentration in the *A. bisporus* group (p=0.081).

267

268 Discussion

269 In this study we have evaluated the effects of fruit juice enriched with the α -
270 glucan fraction from *A. bisporus* on cytokine production patterns by LPS and
271 PHA stimulated PBMCs. The use of fungal β -glucans, and perhaps also of α -
272 glucans, has been proposed to skew the Th1-Th2 balance into the Th1-
273 direction. This would be useful to ameliorate Th2-skewed allergies, and on the
274 other hand promoting Th1 responses in depressed states of immunity. In
275 contrast to the expectation, we here show no skewing into the Th1 direction
276 by *A. bisporus* derived α -glucans. On the contrary, we found that the
277 consumption of α -glucans from *A. bisporus* rather tended to lower the
278 production of the Th1 cytokine IL-12 as compared to control in PHA-induced
279 proliferating PBMCs. IL-12 is produced by monocytes/macrophages and,
280 besides stimulating NK cells, it plays a crucial role in promoting the
281 differentiation of naïve T-cells to a Th1 phenotype. The decreased level of IL-
282 12 after *A. bisporus* is in line with the fact that there was no change in the
283 concentration of the Th1 cytokine IFN γ . In contrast, in mice an elevated IFN γ
284 production by splenocytes after dietary supplementation with *A. bisporus* has
285 been reported (Wu *et al.*, 2007). Moreover, the consumption of an α -glucan-
286 protein complex (94.3% sugar) from another fungal source (*Tricholoma*
287 *matsutake*) recovered the stress-induced decrease in IL-12 serum
288 concentration in mice (Hoshi *et al.*, 2005). This suggests that α -glucans could
289 have *in vivo* effects on Th1 cytokines, at least in mice.

290 Besides IL-12, also the production of IL-10 showed a trend towards
291 decreased production after *A. bisporus* consumption as compared to control

292 drinks. In contrast, Yu *et al.* (Yu *et al.*, 2009) found that oral administration of
293 *A. bisporus* had no effect on *ex vivo* IL-10 concentrations in mice. Moreover,
294 Wu *et al.* (Wu *et al.*, 2007) demonstrated that dietary supplementation of dried
295 *A. bisporus* powder did not affect IL-10 production by mice splenocytes.
296 Therefore, the observation that IL-10 tended to be lower in the *A. bisporus*
297 group is more or less in line with other studies. Finally, the α -glucan fractions
298 from *A. bisporus* did not affect the production of IL-2, IL-13, IL-4 and IL-17.

299 Besides the immune modulating effect of *A. bisporus* α -glucan rich
300 fractions on the Th1-Th2 balance of cytokines produced by PBMCs, we also
301 evaluated the behavior of PBMC towards LPS. We showed that α -glucans
302 from *A. bisporus* reduced TNF α production by LPS stimulated PBMCs as
303 compared to control. This effect is in contrast to our *in vitro* findings in which
304 TNF α secretion by bone marrow-derived macrophages from mice was
305 increased by *A. bisporus* α -glucan extract. This *in vitro* finding was in line with
306 the elevated TNF α and IL-12 secretion by mice macrophages and dendritic
307 cells treated with α -glucans from *Pseudallescheria boydii* *in vitro* (Bittencourt
308 *et al.*, 2006). However, our current finding suggests that in the *in vivo*
309 situation, α -glucans have lost their efficacy to stimulate the response to LPS.

310 There is a vast amount of data from *in vitro* and animal studies showing
311 immune stimulating effects of mushrooms. Comparing our data with that of
312 others, who demonstrated immune stimulating effects, is difficult since earlier
313 studies have used whole mushroom extracts (Wu *et al.*, 2007; Ren *et al.*,
314 2008), while we have specifically evaluated the effect of the α -glucan rich
315 polysaccharide fraction from the mushrooms, which might have contributed to

316 observed differences in results. On the other hand, by comparing results of
317 studies evaluating effects of whole mushroom extracts with our data may
318 provide further insight in effective components since we now know that *in*
319 *vivo*, α -glucans are not effective in promoting Th1 responses by PHA-
320 stimulated PBMCs, as well as stimulating LPS induced cytokine production by
321 PBMCs.

322 The question remains why the *A. bisporus* α -glucan rich fractions did
323 not stimulate human PBMC behavior in response to *ex vivo* LPS and PHA
324 treatments, while there are suggestions from the literature, not only from cell
325 experiments but also from animal studies, that α -glucans have immune
326 stimulating capacity. First, the immunological response of mice and humans
327 differs (Mestas and Hughes, 2004), so the use of different experimental
328 models may have influenced the outcomes. There are several examples in
329 the literature of treatments that were effective in mice, whereas they failed to
330 show effects in humans. For example, IFN γ has been reported to exacerbate
331 disease in an experimental autoimmune model for multiple sclerosis (MS) in
332 mice (Heremans *et al.*, 1996), whereas human clinical trials showed a
333 beneficial effect (Panitch *et al.*, 1987). Additionally, more knowledge regarding
334 intestinal α -glucan handling is essential to explain the lack of effect. When the
335 α -glucans are orally administered, the question arises whether they are
336 absorbed in the gastrointestinal tract and reach the PBMCs as such. For β -
337 glucans it has been demonstrated that mice intestinal macrophages (Hong *et*
338 *al.*, 2004) and enterocytes (presumably Microfold (M)-cells) (Saegusa *et al.*,
339 2004; Rice *et al.*, 2005) can take up and distribute them after oral intake.
340 Whether this also holds for α -glucans needs to be determined. On the other

341 hand, it might be that absorption of the α -glucans is not crucial to exert their
342 effects since intestinal dendritic cells sense the intestinal lumen and by this
343 deliver fragments of the α -glucans to the underlying cells of the intestinal
344 immune system (Rescigno *et al.*, 2001; Niess and Reinecker, 2005).
345 Consequently, activated intestinal lymphocytes may migrate to the mesenteric
346 lymph nodes and come back via lymphatic vessels and blood to intestinal
347 lamina propria. Hypothetically, as food antigen specific T cells have been
348 reported in peripheral blood (Kantele *et al.*, 1999; Eigenmann, 2002), the
349 isolated PBMCs might contain α -glucan activated lymphocytes that normally
350 would return to the intestine. Nevertheless, we did not observe a clear effect
351 on cytokine production by isolated PBMCs, suggesting that if lymphocytes are
352 activated by the mushroom α -glucans, these effects remained local. However,
353 we only analyzed effects in circulating PBMCs and we did not evaluate the
354 intestinal immune response as such. Besides the idea that the immune
355 modulating effects only occurred locally, a final explanation for the lack of a
356 clear immune modulating effect of the *A. bisporus* derived α -glucans might
357 relate to the comparable structure with amylose or pectin. *In vivo* these
358 compounds can be digested by α -amylases present in saliva and pancreatic
359 juice. Furthermore, we investigated the effect of 90% pure α -glucan extract,
360 which does not rule out that other components of *A. bisporus*, like β -glucans,
361 can have an immune stimulating effect. They were, however, only in minor
362 fractions present in our drinks.

363 Safety of the mushroom α -glucan enriched drinks is important as three
364 cases of cancer patients who consumed extracts from another edible
365 mushroom (*Agaricus blazei*) for several days to months during conventional

366 chemotherapy reported severe hepatic damage (Mukai *et al.*, 2006). We
367 found no effects of the *A. bisporus* α -glucan drinks on parameters reflecting
368 liver and kidney function. We even observed a trend towards a lower
369 concentration of creatinine in the *A. bisporus* group as compared to the
370 control group. Therefore we conclude that in this population of healthy
371 subjects with slightly elevated serum total cholesterol concentrations,
372 consumption of 5 g (dry matter) of a α -glucan rich polysaccharide fraction
373 does not affect liver or kidney function, at least within this 5-weeks follow up
374 period.

375 In summary, this is the first large-scale placebo controlled intervention
376 study in humans to evaluate the immune modulating effects of α -glucan from
377 *A. bisporus*. In contrast to the expected immune stimulation into a Th1
378 direction, we here demonstrated no obvious Th1-Th2 skewing effects of α -
379 glucans from *A. bisporus* by PHA-stimulated PBMCs as compared to the
380 control group. Furthermore, we found that the consumption of α -glucans from
381 *A. bisporus* lowers TNF α production by LPS stimulated PBMCs as compared
382 to the control group. The absence of strong immune modulating effect on
383 PBMCs might relate to the comparable structure of α -glucan with amylose or
384 pectin. It is known that these compounds can be digested *in vivo* by α -
385 amylases that are present in saliva and pancreatic juice. Therefore, our
386 current finding suggests that in the *in vivo* situation, α -glucans have lost their
387 efficacy to stimulate the systemic immune response observed in **our *in vitro***
388 **mouse model**. Furthermore, it cannot be excluded that α -glucans have only

389 local effects in the intestine, which are not reflected in systemic PBMC
390 behavior.

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397 financial support and delivering the drinks.

398

399 **Conflict of interest**

400 Leo van Griensven has financial holdings in Innerlife. The other authors
401 declare no conflict of interest.

402

403 **References**

- 404 Bittencourt, VCRT FigueiredoRB da SilvaDS Mourao-SaPL FernandezGL
405 Sasaki *et al.* (2006). An alpha-glucan of *Pseudallescheria boydii* is involved
406 in fungal phagocytosis and Toll-like receptor activation. *J Biol Chem* 281,
407 22614-23.
- 408 Borchers, ATCL Keen and ME Gershwin (2004). Mushrooms, tumors, and
409 immunity: an update. *Exp Biol Med (Maywood)* 229, 393-406.
- 410 Brown, GDJ HerreDL WilliamsJA WillmentAS Marshall and S Gordon (2003).
411 Dectin-1 mediates the biological effects of beta-glucans. *J Exp Med* 197,
412 1119-1124.
- 413 Chen, J and R Seviour (2007). Medicinal importance of fungal beta-(1-->3),
414 (1-->6)-glucans. *Mycol Res* 111, 635-52.
- 415 Eigenmann, PA (2002). T lymphocytes in food allergy: overview of an intricate
416 network of circulating and organ-resident cells. *Pediatr Allergy Immunol* 13,
417 162-71.
- 418 Heremans, HC DillenM GroenenE Martens and A Billiau (1996). Chronic
419 relapsing experimental autoimmune encephalomyelitis (CREAE) in mice:
420 enhancement by monoclonal antibodies against interferon-gamma. *Eur J*
421 *Immunol* 26, 2393-8.
- 422 Hong, FJ YanJT BaranDJ AllendorfRD HansenGR Ostroff *et al.* (2004).
423 Mechanism by which orally administered beta-1,3-glucans enhance the
424 tumoricidal activity of antitumor monoclonal antibodies in murine tumor
425 models. *J Immunol* 173, 797-806.

426 Hoshi, HY YagiH IijimaK MatsunagaY Ishihara and T Yasuhara (2005).
427 Isolation and characterization of a novel immunomodulatory alpha-glucan-
428 protein complex from the mycelium of *Tricholoma matsutake* in
429 basidiomycetes. *J Agric Food Chem* 53, 8948-56.

430 Kantele, AJ ZivnyM HakkinenCO Elson and J Mestecky (1999). Differential
431 homing commitments of antigen-specific T cells after oral or parenteral
432 immunization in humans. *J Immunol* 162, 5173-7.

433 Lin, YLSS LeeSM Hou and BL Chiang (2006). Polysaccharide purified from
434 *Ganoderma lucidum* induces gene expression changes in human dendritic
435 cells and promotes T helper 1 immune response in BALB/c mice. *Mol*
436 *Pharmacol* 70, 637-44.

437 Lull, CHJ Wichers and HF Savelkoul (2005). Antiinflammatory and
438 immunomodulating properties of fungal metabolites. *Mediators Inflamm* 2005,
439 63-80.

440 Mestas, J and CC Hughes (2004). Of mice and not men: differences between
441 mouse and human immunology. *J Immunol* 172, 2731-8.

442 Mukai, HT WatanabeM Ando and N Katsumata (2006). An alternative
443 medicine, *Agaricus blazei*, may have induced severe hepatic dysfunction in
444 cancer patients. *Jpn J Clin Oncol* 36, 808-10.

445 Niess, JH and HC Reinecker (2005). Lamina propria dendritic cells in the
446 physiology and pathology of the gastrointestinal tract. *Curr Opin Gastroenterol*
447 21, 687-91.

448 Panitch, HSRL HirschAS Haley and KP Johnson (1987). Exacerbations of
449 multiple sclerosis in patients treated with gamma interferon. *Lancet* 1, 893-5.

450 Ren, ZZ GuoSN Meydani and D Wu (2008). White button mushroom
451 enhances maturation of bone marrow-derived dendritic cells and their antigen
452 presenting function in mice. *J Nutr* 138, 544-50.

453 Rescigno, MM UrbanoB ValzasinaM FrancoliniG RottaR Bonasio *et al.*
454 (2001). Dendritic cells express tight junction proteins and penetrate gut
455 epithelial monolayers to sample bacteria. *Nat Immunol* 2, 361-7.

456 Rice, PJEL AdamsT Ozment-SkeltonA GonzalesMP GoldmanBE Lockhart *et*
457 *al.* (2005). Oral delivery and gastrointestinal absorption of soluble glucans
458 stimulate increased resistance to infectious challenge. *J Pharmacol Exp Ther*
459 314, 1079-1086.

460 Saegusa, SM TotsukaS Kaminogawa and T Hosoi (2004). *Candida albicans*
461 and *Saccharomyces cerevisiae* induce interleukin-8 production from intestinal
462 epithelial-like Caco-2 cells in the presence of butyric acid. *FEMS Immunol*
463 *Med Microbiol* 41, 227-235.

464 Saito, KT YajimaH NishimuraK AibaR IshimitsuT Matsuguchi *et al.* (2003).
465 Soluble branched beta-(1,4)glucans from *Acetobacter* species show strong
466 activities to induce interleukin-12 in vitro and inhibit T-helper 2 cellular
467 response with immunoglobulin E production in vivo. *J Biol Chem* 278, 38571-
468 8.

469 Suzuki, YY AdachiN Ohno and T Yadomae (2001). Th1/Th2-Balancing
470 immunomodulating activity of gel-forming (1-->3)-beta-glucans from fungi. *Biol*
471 *Pharm Bull* 24, 811-9.

472 Takimoto, HH KatoM Kaneko and Y Kumazawa (2008). Amelioration of
473 Skewed Th1/Th2 Balance in Tumor-Bearing and Asthma-Induced Mice by

474 Oral Administration of *Agaricus blazei* Extracts. *Immunopharmacol*
475 *Immunotoxicol* 1-14.

476 Volman, JJJPGF HelsperS WeiJJJ BaarsLJLD van GriensvenASM
477 Sonnenberg *et al.* (2009). Effects of mushroom derived beta-glucan-rich
478 polysaccharide extracts on NO production by bone marrow-derived
479 macrophages and NF-kappaB transactivation in Caco-2 reporter cells: can
480 effects be explained by structure? *Mol Nutr Food Res* in press.

481 Wasser, SP (2002). Medicinal mushrooms as a source of antitumor and
482 immunomodulating polysaccharides. *Appl Microbiol Biotechnol* 60, 258-74.

483 Wei, SJJPGF Helsper and LJLD Van Griensven (2008). Phenolic compounds
484 present in medicinal mushroom extracts generate reactive oxygen species in
485 human cells *in vitro*. *Int J Med Mush* 10, 1-13.

486 Wei, S and LJ Van Griensven (2008). Pro- and antioxidative properties of
487 medicinal mushroom extracts. *Int J Med Mushr* 10, 315-324.

488 Wu, DM PaeZ RenZ GuoD Smith and SN Meydani (2007). Dietary
489 supplementation with white button mushroom enhances natural killer cell
490 activity in C57BL/6 mice. *J Nutr* 137, 1472-7.

491 Yu, SV WeaverK Martin and MT Cantorna (2009). The effects of whole
492 mushrooms during inflammation. *BMC Immunol* 10, 12.

493 Zhang, P and PC Cheung (2002). Evaluation of sulfated *Lentinus edodes*
494 alpha-(1-->3)-D-glucan as a potential antitumor agent. *Biosci Biotechnol*
495 *Biochem* 66, 1052-6.

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499 **Legends to the figure**

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501 **Figure 1.** Effects of α -glucan consumption (5 g/d) from *A. bisporus* on TNF α
502 concentration after ex vivo LPS stimulation of PBMCs. PBMCs were

503 stimulated for 4h with 10 ng/mL LPS from *E Coli* 055:B5. * p=0.017 vs control

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507 **Tables**

508 **Table 1.** Baseline characteristics of the subjects before start of the study.

	Control	<i>A. bisporus</i>
Age (years)	57 ± 9	57 ± 7
Sex (males/females)	17/13	15/11
Smokers (yes/no)	2/28	2/24
BMI (kg/m ²)	25.4 ± 3.6	26.4 ± 2.8
Total cholesterol (mmol/L)	6.55 ± 0.89	6.65 ± 0.70
Systolic blood pressure (mmHg)	129 ± 12	127 ± 12
Diastolic blood pressure (mmHg)	84 ± 7	84 ± 7

509 Values are means ± SD.

510 **Table 2.** Effects of α -glucan consumption (5 g/d) from *A. bisporus* on median
 511 IL-1 β , IL-6 and TNF α concentration after ex vivo LPS stimulation of PBMCs.

	Control (N=30)		<i>A. bisporus</i> (N=26)	
IL-1β (pg/mL)				
Run-in	391	(151 to 1189)	488	(217 to 1030)
Test period	455	(126 to 1427)	425	(158 to 1364)
Change	15	(-412 to 346)	-81	(-387 to 334)
IL-6 (pg/mL)				
Run-in	1760	(535 to 4093)	2249	(466 to 4108)
Test period	2310	(776 to 5378)	2334	(894 to 4710)
Change	396	(-695 to 1768)	212	(-834 to 1633)
TNFα (pg/mL)				
Run-in	3053	(885 to 8203)	3465	(944 to 6688)
Test period	3795	(895 to 8414)	3677	(863 to 7499)
Change	792	(-1071 to 1675)	249 ^a	(-656 to 2025)

512 Values are medians with ranges. PBMCs were stimulated for 4h with 10
 513 ng/mL LPS from *E Coli* 055:B5. ^ap=0.017 vs control
 514

515 **Table 3.** Effects of α -glucan consumption (5 g/d) from *A. bisporus* on median
 516 IFN γ , IL-12, IL-2, IL-13, IL-4, IL-10 and IL-17 concentration after *ex vivo* PHA
 517 stimulation of PBMCs.

	Control (N=25)		A. bisporus (N=23)	
<u>Th1 cytokines</u>				
IFNγ (pg/mL)				
Run-in	2549	(918 to 9444)	2432	(525 to 7484)
Test period	2439	(453 to 7935)	1882	(667 to 9036)
Change	-291	(-4068 to 1392)	141	(-1534 to 3106)
IL-12 (pg/mL)				
Run-in	59	(37 to 108)	57	(47 to 148)
Test period	55	(30 to 125)	55	(24 to 106)
Change	4	(-51 to 53)	-10 ^a	(-79 to 37)
IL-2 (pg/mL)				
Run-in	6362	(1442 to 18194)	8618	(741 to 31607)
Test period	6206	(1513 to 15881)	4776	(600 to 28082)
Change	-937	(-8112 to 5177)	-2073	(-10866 to 7649)
<u>Th2 cytokines</u>				
IL-13 (pg/mL)				
Run-in	3185	(946 to 6631)	3064	(1323 to 7254)
Test period	3019	(942 to 6450)	3157	(1258 to 7980)
Change	-181	(-1835 to 1857)	-201	(-3281 to 1147)
IL-4 (pg/mL)				
Run-in	179	(94 to 468)	190	(90 to 668)
Test period	154	(76 to 453)	180	(56 to 418)
Change	-12	(-205 to 171)	-12	(-292 to 73)
<u>Other cytokines</u>				
IL-10 (pg/mL)				
Run-in	1310	(399 to 3095)	1106	(618 to 2783)
Test period	1422	(455 to 3956)	1057	(631 to 2127)
Change	56	(-644 to 1343)	-159 ^b	(-1454 to 918)
IL-17 (pg/mL)				
Run-in	993	(402 to 3651)	1414	(451 to 2921)
Test period	741	(391 to 2470)	934	(259 to 1902)
Change	-159	(-1362 to 487)	-246	(-1958 to 256)

518 Values are medians with ranges. PBMCs were stimulated for 52h with 50
 519 μ g/mL PHA. ^a p=0.059 vs control, ^b p=0.053 vs control

520

521 **Table 4.** Effects of α -glucan consumption (5 g/d) from *A. bisporus* on median
 522 serum creatinine, alanine transaminase (ALAT), aspartate aminotransferase
 523 (ASAT), γ -glutamyl transpeptidase (γ -GT), alkaline phosphatase, and total
 524 bilirubin concentrations.

	Control (N=30)	A. bisporus (N=26)
Creatinine ($\mu\text{mol/L}$)		
Run-in	85 (63 to 136)	85 (61 to 119)
Test period	86 (60 to 140)	80 (60 to 125)
Change	-1 (-12 to 15)	-4 ^a (-32 to 12)
ALAT (U/L)		
Run-in	26 (12 to 58)	27 (18 to 54)
Test period	26 (10 to 77)	29 (16 to 81)
Change	2 (-12 to 45)	1 (-12 to 52)
ASAT (U/L)		
Run-in	21 (11 to 37)	22 (12 to 32)
Test period	24 (9 to 43)	24 (10 to 41)
Change	2 (-6 to 26)	1 (-11 to 20)
γ-GT (U/L)		
Run-in	22 (12 to 46)	27 (15 to 75)
Test period	24 (14 to 66)	25 (14 to 76)
Change	-1 (-9 to 20)	0 (-13 to 28)
Alkaline phosphatase (U/L)		
Run-in	61 (20 to 101)	69 (45 to 133)
Test period	60 (24 to 101)	73 (48 to 130)
Change	4 (-9 to 15)	3 (-24 to 30)
Bilirubin ($\mu\text{mol/L}$)		
Run-in	14 (7 to 33)	12 (8 to 25)
Test period	12 (5 to 25)	12 (6 to 28)
Change	-2 (-10 to 5)	-1 (-10 to 6)

525 Values are medians with ranges.^a p=0.081 vs control

