In vitro and *in vivo* testing of the hypocholesterolemic activity of ergosterol- and β-glucan-enriched extracts obtained from shiitake mushrooms (*Lentinula edodes*)

Diego Morales*[1], Eva Tejedor-Calvo[1], Noelia Jurado-Chivato[1], Gonzalo Polo[2], María Tabernero[2,3], Alejandro Ruiz-Rodríguez[1], Carlota Largo[2], Cristina Soler-Rivas[1].

[1]Department of Production and Characterization of Novel Foods, Institute of Food Science Research - CIAL (UAM+CSIC), C/ Nicolas Cabrera 9, Campus de Cantoblanco, Universidad Autónoma de Madrid, 28049 Madrid, Spain.

[2]Departament of Experimental Surgery, Research Institute Hospital La Paz (IdiPAZ), Paseo de la Castellana 261, 28046 Madrid, Spain

[3]Department of Innovation in Precision Nutrition, Imdea-Food Institute, CEI UAM+CSIC, Carretera de Cantoblanco 8, 28049 Madrid, Spain

* Corresponding author: diego.morales@uam.es

15   **Abstract**

16   A supercritical extraction plant (with a 6 L extraction cell) was successfully used to obtain

17   ergosterol-enriched extracts from Lentinula edodes (40 ºC, 225 bar, 1-5h, 20 L/h recirculated CO_2).

18   Ergosterol (ERG) and the SFE extract (SFE) with the highest ergosterol concentration were also

19   microemulsified and submitted to in vitro digestion to study their ability to displace cholesterol

20   from dietary mixed micelles (DMMs). ERG was also mixed with a β-glucan-enriched (33.5%)

21   extract (BGE) from L. edodes to investigate synergies. Results indicated that they all (including

22   BGE without ERG) could reduce cholesterol levels in DMMs. However, when ERG and SFE were

23   administrated to mice simultaneously with a hypercholesterolemic diet, no significant differences in

24   serum cholesterol levels were detected compared to control. Only when BGE was administrated to

25   another mice model after the hypercholesterolemia was previously induced, significant reduction of

26   cholesterol was noticed.

1. Introduction

High levels of total cholesterol (TC) and particularly LDL-cholesterol constitute one of the most relevant risk factors of cardiovascular diseases that are still leading the causes of death, particularly in developed countries. Before the treatment with therapeutic drugs, for patients still with milder hypercholesterolemia, regular consumption of specific functional foods might be more desirable. Nowadays, products including phytosterols or cereal β-glucans bear a health claim as hypocholesterolemic products approved by most of regulatory institutions such as European Food Safety Authority (EFSA), Food and Drugs Administration (FDA), etc. and they are easily found in the supermarkets, however, novel or/and more effective products are being developed.[1,2]

Edible mushrooms were pointed as one of those foods with potential hypocholesterolemic activities.[3] They contain fungal sterols that (as noticed for phytosterols) might displace cholesterol from dietary mixed micelles (DMMs).[4] Dietary cholesterol is mainly absorbed by intestinal enterocytes when incorporated into these small micelles together with the rest of lipid compounds. They are formed during digestion when pancreatic lipases, bile acids, lecithin and other molecules transform the dietary fat into oily drops and large vesicles that reduced their size until micelles generating a particular emulsion where only those micelles with the optimal dimensions are incorporated into the cell membranes and then, they are called DMMs. The larger size vesicles are usually excreted together with the precipitated compounds that are eliminated from the micelles. The lipids provided by DMMs are only assimilated by enterocytes when specific transporters recognize them and transfer them into the endoplasmic reticulum. Apparently, for cholesterol intake, the Niemann-Pick C1-like 1 protein is necessary and it showed high affinity for this molecule so non-cholesterol sterols are incorporated in very low amounts compared to cholesterol (approx. 2 – 5% vs. 60%).[5,6]

The bioavailability of fungal sterols such as ergosterol could be enhanced by loading it into specific microemulsions improving, for instance, its anti-tumor properties.[7] Microemulsions are thermodynamically stable nanometer systems used to stabilize compounds difficult to solubilize in

physiological fluids. Besides cholesterol displacers from DMM, plant or fungal sterols were described as modulators of genes involved in the cholesterol metabolism (SREBP, NR1H3 (LXR) FDFT1 (SQS), etc.), as inhibitors of specific enterocytic enzymes needed for cholesterol absorption and transport (SOAT, MTT, APO48 etc.), as liver X receptor (LXR) agonists, etc.[8-10] However, at the present, it is still unclear whether plant/fungal sterols should be absorbed in higher concentrations to exert their hypocholesterolemic activities since when taken in large amounts they also induce disorders such as sitosterolemia.[11] Moreover, if they are loaded into microemulsions, they might have more difficulties to be incorporated in the DMMs and hence to displace cholesterol.

On the other hand, the inclusion of fungal β-glucan extracts into ergosterol supplemented food matrices seemed to improve the cholesterol displacement from DMMs.[4] These polymers also showed hypocholesterolemic activities although their mechanisms of action seemed to differ from those postulated for sterols. These polysaccharides showed gel forming properties and they might increase viscosity during digestion stimulating cholesterol excretion through faeces. They were also reported as bile acids scavengers, enhancing the transformation of cholesterol to reestablish their levels. Moreover, β-glucans fermentation by colonic microbiota seemed to stimulate the production of short-chain fatty acids that might block cholesterol biosynthesis by inhibiting the HMGCR, a key enzyme within the cholesterol pathway.[12]

Ergosterol-enriched extracts can be successfully obtained from natural sources such as mushrooms using supercritical CO_2 extraction, *e.g.* from *Lentinula edodes*[13,14], *Agaricus bisporus*[15] or *Agaricus brasiliensis*.[16] The process was more effective than the use of organic solvents or other advanced technologies such as pressurized liquid-extraction or microwave/ultrasound-assisted technologies.[15,17,18] However, the SFE plants utilized in most of the previous publications are usually small or pilot plant scale where extraction conditions can be very easily controlled but lower amounts of extract are obtained compared to plants using higher volume extraction cells. Hence, to obtain extracts in large quantities to be tested in animal or clinical trials, a large-scale SFE plant should be utilized as well as some adjustments should be taken into consideration.

4

80   In this work, a large-scale SFE plant was used to obtain enough amounts of ergosterol-

81 enriched extract from *Lentinula edodes* to carry out *in vitro* and *in vivo* tests to study its

82 hypocholesterolemic activities. The obtained extract was loaded into a microemulsion and mixed

83 with a fungal β-glucan-enriched extract to investigate whether they might enhance or interfere with

84 its activity as cholesterol displacer from DMMs during an *in vitro* digestion model. Two different

85 mice models were also utilized to evaluate the *in vivo* hypocholesterolemic effect of the SFE extract

86 as well as the β-glucan-enriched extract.

87   **2. Materials & Methods**

88 **2.1. Biological material, standards and reagents**

89   Powdered *Lentinula edodes* S. (Berkeley) fruiting bodies (particle size < 0.5 mm, moisture

90 < 5%) were purchased from Glucanfeed S.L. (La Rioja, Spain) and stored in darkness at -20 ℃ until

91 further use. Commercially available lard (Iberian pork lard, 99.7 g /100 g fat) was purchased from a

92 local supermarket and maintained at 4 ℃ until use. All the experiments were performed from the

93 same lot.

94   Solvents as hexane (95%), chloroform (HPLC grade), methanol (HPLC grade) were

95 obtained from LAB-SCAN (Gliwice, Poland) and absolute ethanol from Panreac (Barcelona,

96 Spain). Calcium chloride, hydrochloric acid (37%), pepsin (from porcine pancreatic mucosa),

97 sodium hydroxide, sodium chloride, Trizma base, maleic acid, pancreatin (from porcine pancreas),

98 L-α-phosphatidyl choline (lecithin), Sepharose® 4B, phenol, ascorbic acid, BHT (2,6-Di-tert-butyl-

99 *p*-cresol) and Kolliphor®EL were purchased from Sigma-Aldrich Química (Madrid, Spain) as well

100 as cholesterol (96%) and ergosterol (95%) used as standards. Carbon dioxide (99.99% purity) was

101 supplied by Air-Liquid España, S.A. (Madrid, Spain).

102 **2.2. Supercritical fluid extractions (SFE)**

103   Extractions with CO_2 were carried out in a large scale SFE plant (Zean Consultores S.L.,

104 Madrid, Spain) comprising a 6 L cylinder extraction vessel and two different separators (S1 and S2)

105 of 1.6 L capacity each with independent control of temperature and pressure. A detailed explanation

106 of the experimental device can be found elsewhere.[19] The CO_2 flow was set at 20 L/h and during the

107   total extraction time tested (1 to 5 h) it was recirculated. The temperature was fixed at 40 ºC in the

108   extraction vessel and the separators and the extraction pressure was maintained at 225 bar. Ethanol

109   was not used as cosolvent because previous studies indicated that although higher yields could be

110   obtained, the use of only CO_2 was more selective generating fractions with higher sterols

111   concentrations[15]. The fractions extracted in 2 separators (S1 and S2) were collected with ethanol

112   and immediately submitted to dryness on a rotary vacuum evaporator. Ethanol was utilized because

113   it was used in previous studies[19] since it is an organic solvent easy to evaporate with mild processes

114   and it was able to completely solubilize separated fractions detaching the extracts from separator

115   walls. The dried extracts were stored at -20 ºC until further analysis.

116   **2.3. Preparation of ergosterol and SFE extract microemulsions**

117   Sterol-loaded microemulsions were formulated as indicated by Yi et al. (2012)[7] with slight

118   modifications. Briefly, ergosterol (50 mg) or a selected SFE extract containing 52.5% ergosterol

119   (SFE) (95 mg) were mixed with lard (1 g). Afterwards, Koliphor EL (21% w/w) and 5% ethanol

120   were mixed thoroughly and stirred as surfactant-cosurfactant solution and mixed with 3% of the

121   supplemented lards. Then, water (71%) was added dropwise into the oily mixtures with gentle

122   stirring to generate microemulsions. Two different microemulsions, MERG (microemulsified

123   ergosterol) and MSFE (microemulsified SFE extract) were prepared and immediately submitted to

124   the *in vitro* digestion model.

125   **2.4. Preparation of a β-glucan-enriched (BGE) extract**

126   A β-glucan-enriched extract (BGE) was prepared by mixing different polysaccharides

127   fractions with hypocholesterolemic activities such as a fraction (2.5%) containing water soluble β-

128   glucans (named ExA by Morales et al. (2018)[20], a fraction (26%) extracted with hot water (98 ºC),

129   filtered through a multichannel ceramic membrane (Ceramem Corporation, Waltham, USA) and

130   concentrated with a spiral wound Nanomax50 membrane (Millipore, Bedford, USA) (called RF2 in

131   Morales et al. (2019)[21]) and the remaining fraction (71.5%) containing mainly chitins and insoluble

132   β-glucans.[21] The fractions were lyophilized, pooled together and stored at -20ºC until further use.

133   The resulting mixture contained 33.5% (w/w dry weight) β-glucans and 0.23% ergosterol

134   (determined as Morales et al. (2018)[20]).

135   **2.5. *In vitro* digestion and isolation of the dietary mixed micelles (DMM)**

136   Lard supplemented with cholesterol (25 mg/g) was used as food matrix to evaluate the

137   hypocholesterolemic activity of the different extracts within an *in vitro* digestion model as indicated

138   by Gil-Ramirez et al. (2014)[4]. The hypercholesterolemic lard was mixed with ergosterol (ERG) (50

139   mg/g), MERG (32.3 g/g), SFE (95 mg/g) or MSFE (32.3 g/g) as ergosterol-containing formulations

140   and/or BGE extract (149 mg/g) as β-glucan plus ergosterol-containing extract (the indicated

141   concentrations were adjusted to include 50 mg/g ergosterol and/or β-glucans in the food matrix).

142   The mixtures were gently stirred at their melting temperature until complete incorporation of the

143   supplements into the lipidic matrices.

144   The supplemented food matrices (1 g) were submitted to *in vitro* digestion following the

145   procedure described by Gil-Ramirez et al. (2014).[4] Afterwards, the fraction containing the dietary

146   mixed micelles (DMM) was isolated using a Sepharose®4B column with 0.15M NaCl/16 mM bile

147   salts as mobile phase as indicated by their intermicellar bile salt concentration (IMBC). The DMM

148   fractions (16 mL) were identified because their cholesterol concentration (determined by an

149   enzymatic SpinReact cholesterol quantification kit (SpinReact SAU, Girona, Spain) co-eluted with

150   their phospholipids concentration (determined using an enzymatic Wako kit (Wako, Madrid,

151   Spain)) at the proper elution volume (Gil-Ramirez et al., 2014).[4]

152   **2.6. Sterols quantification by GC-MS-FID**

153   Sterols were extracted from the samples and quantified following the procedure described

154   by Gil-Ramirez et al. (2013).[15] The unsaponified fractions were injected into an Agilent 19091S-

155   433 capillary column (30 m x 0.25 mm ID and 0.25 μm phase thickness). The column was

156   connected to a 7890A System gas chromatograph (Agilent Technologies, Santa Clara, USA)

157   including a G4513A auto injector and a 5975C triple-axis mass spectrometer detector. The injector

158   and detector conditions, as well as the column temperature program, were those described by Gil-

159 Ramirez et al. (2013).[15] Cholesterol and ergosterol were used as standards and hexadecane (10%

160 v/v) as internal standard.

161   GC-MS database identified the obtained peaks in concordance with previous studies.[15,22,23]

162 The major detected sterols were cholesterol (RT = 11.9 min), ergosterol (ergosta-5,7,22-trien-3β-ol)

163 (RT= 12.6 min), ergosta 7,22-dienol (RT =12.8 min), ergosta-5,7-dienol (RT = 13.1 min) and

164 ergosta-7-enol (fungisterol) (RT= 13.3 min).

165 **2.7. Animal trials**

166   Male mice C57/BL6JRj (6 weeks; Janvier SAS, Le Genest-Saint-Isle, France) were

167 maintained in temperature-, humidity- and light-controlled conditions (24±2°C, 40-60% humidity,

168 12:12 hour light: dark cycle) and had free access to water and food. Two different experiments were

169 carried out and both were approved by the Institutional Animal Welfare and Ethics Committee of

170 La Paz University Hospital (Madrid, Spain) according to the current Spanish and European

171 legislation (RD53/2013 and EU 63/2010).

172   In the first experiment, 35 mice were randomly divided in four groups. Group NC (normal

173 control, n = 5) was fed a control diet (standard diet, Safe Rodent Diet, A04, Augy, France), group

174 HC1 (high cholesterol, n = 10) was administrated a high fat hypercholesterolemic diet (HFHD)

175 (standard diet supplemented with 1.25% cholesterol, 0.5% cholic acid and 12% lard), group ERG (n

176 = 10) was given the HFHD supplemented with ergosterol (0.45%) and group SFE (n = 10) was fed

177 HFHD supplemented with SFE extract (up to 0.45% ergosterol in the diet). Blood samples were

178 obtained by mandibular puncture at the beginning and the end of the study (5 weeks) and plasma

179 samples were stored at -20 ºC until further use.

180   In the second experiment, 20 mice were fed the standard diet supplemented with 1.25%

181 cholesterol and 0.5% cholic acid and the blood samples were obtained at the beginning and 3 weeks

182 after the feeding started. After 4 weeks, animals were randomly divided in two groups (n = 10), one

183 continued with the same diet and it was used as control (HC2) and the other (BGE) was

184 administrated the diet supplemented with 6% BGE. Both were fed during 5 weeks.

185   After the feeding periods, mice from both experiments were killed by intracardiac

186 exsanguination under anesthesia with 1.5 % isoflurane, and plasma was collected and stored at −20

187 °C before use. Liver, fat, kidney and samples were removed and immediately frozen in liquid

188 nitrogen and stored at −80 °C. Plasma levels of total cholesterol, triglycerides, HDL-cholesterol and

189 LDL-cholesterol were measured using a Covas C311 Autoanalyzer (Roche, Spain).

190   **2.9. Statistical analysis**

191   Differences were evaluated at 95% confidence level ($P \leq 0.05$) using one-way analysis of

192 variance (ANOVA) followed by Tukey's Multiple Comparison test. Statistical analysis was

193 performed using SPSS V.13.0 software (SPSS Institute Inc., Cary, NC) and GraphPad Prism

194 version 5.01 (GraphPad Software, San Diego, CA, USA).

195
196   **3.   Results and discussion**
197
198   **3.1 Upscaling of SFE extractions**

199   Large-scale supercritical CO_2 extractions were carried out to scale up the production of SFE

200 extracts from shiitake mushrooms since similar extracts obtained from *Agaricus bisporus* decreased

201 the TC/HDL ratio in hypercholesterolemic mice when applied at low concentrations. In this sense,

202 Gil-Ramirez et al. (2016)[9] concluded that, in order to improve its hypocholesterolemic activity,

203 higher concentrations of SFE extracts should be utilized for *in vivo* testing.

204   Extraction yields were increasing with extraction time up to 3h reaching values of 0.53% taking

205 into consideration the material collected in both separators (S1+S2) (Figure 1). However, when

206 longer extraction times were tested (up to 5h) only a slight increase was noticed (0.56 % yield) after

207 2h more of extraction. The S1 separator collected more material than S2 (2.3-2.8 folds)

208 independently of the extraction time. Previous works carrying out SFE extractions from shiitake

209 mushrooms with a pilot scale plant and similar conditions (225 bar, 35ºC, 3 h) showed higher yields

210 (1.25%).[14] Those yields could be improved in pilot-plants by increasing temperature (2.3% at 225

211 bar and 75 ºC) or pressure (2.1% at 350 bar and 40 ºC). Nevertheless, other authors obtained similar

9

212   values in shiitake SFE at 40 ℃ during 3 h under 200 and 250 bar (0.65 and 0.81%, respectively)

213   using smaller plant with a 100 mL extraction cell.[13]

214   The obtained SFE extracts contained up to 53% ergosterol (Table 1), similar values than those

215   indicated by Gil-Ramirez et al. (2013)[15] for *Agaricus bisporus* but significantly higher than others

216   noticed for *L. edodes* using a smaller capacity pilot plant.[14] Results showed that, although the yield

217   in this large-scale plant was lower, the extraction was apparently more selective toward sterols than

218   when using a pilot plant. Moreover, when the ergosterol content was determined in the extracts

219   collected in both separators, the S1 extracts exhibited higher cholesterol concentrations (41-53%)

220   than S2 extracts (19-26%) and statistically significant differences were found in S1 between 1 and 5

221   h of extraction time suggesting than time could influence ergosterol enrichment of the extracts.

222   Ergosterol derivatives were also detected in the extracts, although at lower concentrations

223   (Table 1). Fungisterol concentrations were higher than ergosta5,7-dienol and ergosta7,22-dienol and

224   it was distributed within the separators in a similar ratio to ergosterol. However, ergosta7,22-dienol

225   was specifically recovered in S2 and ergosta5,7-dienol values showed no differences between

226   separators. Thus, the extract containing the larger amount of fungal sterols (58.7%) obtained after

227   5h in S1 was selected as SFE extract to carry out further experiments.

228   **3.2. *In vitro* testing of the hypocholesterolemic properties of obtained extracts**

229   Previous *in vitro* studies indicated that ergosterol and particularly a SFE extract containing

230   fungal sterols from *A. bisporus,* were as effective as β-sitosterol in the displacement of cholesterol

231   from DMM when incorporated in a food matrix such as lard.[4] In order to test the ability of the SFE

232   extract obtained from *L. edodes,* the same *in vitro* digestion model was reproduced. Results

233   indicated that the DMM fraction generated after digestion of lard supplemented with cholesterol and

234   ergosterol (ERG) contained approx. 63% less cholesterol than when only cholesterol was added

235   (Figure 2). This reduction was slightly higher than the 49% noticed by Gil-Ramirez et al. (2014)[4]

236   but the effect reported when the SFE extracts were added, was almost the same (69% and 67%,

237   respectively) indicating that, independently of the mushroom utilized, the SFE extracts were able to

238 reduce cholesterol levels in the DMM fraction (that is the one usually absorbed by intestinal

239 enterocytes).

240 Moreover, according to previous studies, the bioavailability of phytosterols, as well as other

241 non-cholesterol sterols was very limited[8] but other reports indicated that ergosterol bioavailability

242 might be enhanced by loading them into microemulsions.[7] However, if sterols are incorporated into

243 artificial vesicles and these are incorporated into food matrices, they might be more bioavailable but

244 perhaps they are not able to act as cholesterol displacers in the DMMs. Therefore, ergosterol and the

245 SFE extract were emulsified (MERG and MSFE, respectively), supplemented to the

246 hypercholesterolemic lard and digested to investigate the content of the DMM fractions generated.

247 Results indicated that both extracts could displace cholesterol from the generated DMMs as their

248 corresponding non-microemulsified extracts.

249 Other reports suggested that the presence of β-glucans extracts obtained from *Pleurotus*

250 *ostreatus* in the food matrix functionalized with ergosterol reduced the cholesterol content of the

251 generated DMMs approx. 52% more than if only the sterol was added.[4] When similar food mixture

252 was prepared but using a β-glucan-enriched extract obtained from *L. edodes* (BGE+ERG), a lower

253 cholesterol level was noticed in the DMM fraction generated than in the DMM control (CH),

254 however, no significantly different values were noted compared to the DMM fraction generated

255 when the food matrix was supplemented only with ERG. Differences with previous investigations

256 might be due to the structural differences between the polysaccharides from both mushroom species

257 or because of their specific extract compositions. Surprisingly, when the BGE extract was also

258 tested without any sterol addition, significant cholesterol reduction was also noticed (33.7%) in its

259 DMM fraction compared to the control. Since the ergosterol levels in BGE extract were really low

260 (0.23%), the noticed hypocholesterolemic activity might be because of its high β-glucans content

261 (33.5%). These polymers were able to scavenge other small molecules such as bile acids or

262 cholesterol in their structures.[24,25] In fact, it was suggested as their potential mechanism to lower

263 cholesterol levels in serum[12] although there might be others.[26]

264   Ergosterol was also detected in the generated DMM fractions but it was always incorporated in

265   lower concentrations than cholesterol (on average 58% less) corroborating previous results.[4]

266   Moreover, no significant differences were noticed between the different tested extracts suggesting

267   that its incorporation in the DMM was more related to the ergosterol content of the food matrix than

268   to the presence of other components in the mixture (such as *i.e.* β-glucans, surfactants, etc.) because

269   all the extracts were supplemented to the hypercholesterolemic lard in different concentrations to

270   ensure the same ergosterol content.

271   **3.3.*In vivo* testing of the hypocholesterolemic properties of obtained extracts**

272   Animal studies were carried out to confirm the hypocholesterolemic activity noticed *in vitro* for

273   the extracts. However, in order to minimize the number of mice, only ergosterol as standard

274   compound and the SFE extract were tested since no significant differences were found with the

275   MERG or MSFE in their capacity to displace cholesterol from DMMs. The MSFE preparation

276   included more processing that might increase the cost if up scaled. Moreover, the administration of

277   the preparations was carried out at the same time than the induction of hypercholesterolemia to

278   study their potential as preventive more than palliative formulations since the latter influence was

279   already investigated for a SFE extract obtained from *A. bisporus*.[9]

280   The mice groups treated with normal diet (NC) or hypercolesterolemic diet (HC1) used as

281   control as well as the ERG and SFE groups showed at the beginning of the experimental period

282   similar values of total cholesterol (TC) (on average 91.4±10.8 mg/dL), HDL (1.9±0.7 mmol/L),

283   LDL (0.2±0.1 mmol/L), triglycerides (TG) (158±68.8 mg/dL), AST (74.8±36.1 UI/dL), ALT

284   (18.0±8.1 UI/dL) and glucose 50.0±27.5 (mg/dL). However, after 5 weeks only the NC group

285   maintained those levels and the rest of groups increased their TC levels with non-significant

286   differences between HC1 (230.3±67.1 mg/dL), ERG (277.3±35.5 mg/dL) or SFE (262±52.6 mg/dL)

287   groups. The increase noticed in ERG group was more influenced by a high LDL value (5.7±1.0

288   while HC1 showed 4.6±1.7 mmol/L) and the one noticed in the SFE group by a high HDL value

289   (3.2±1.0 while HC1 showed 2.2±0.3 mmol/L), although differences in both parameters were also

290 insignificant. Similarly, the TC and glucose values increased in all the groups (40.5 to 45.6 mg/dL

291 and 155.5 to 192.7 mg/dL, respectively). Therefore, the ergosterol containing preparations showed

292 no hypocholesterolemic activity *in vivo*. These results differed from recent studies carried out in rats

293 fed simultaneously a hypercholesterolemic diet supplemented with 0.5% or 1.5% ergosterol for 8

294 weeks[27] or previous tests carried out in mice but where firstly hypercholesterolemia was induced

295 and then treated with ergosterol-containing preparations obtained from *A. bisporus*.[9]

296   Thus, since the BGE extracts also showed hypocholesterolemic activity *in vitro* but their

297 mechanism of action might be different than fungal sterols, another *in vivo* experiment was carried

298 out to investigate whether the β-glucan-enriched extract was more effective than fungal sterols. This

299 time, the experimental setting was changed and firstly, mice were fed a hypercholesterolemic diet

300 and then they were administrated the BGE extract together with the unhealthy diet to investigate

301 their effect as palliative compounds. Results indicated that administration of the standard diet

302 supplemented with cholesterol and cholic acid for 3 weeks increased TC, HDL and LDL levels in

303 all the mice inducing hypercholesterolemia (Table 2). The glucose concentration and hepatic

304 enzymes activities were also influenced and increased their values remaining only the TG amounts

305 unchanged. After 4 weeks, HC2 group continued with the hypercholesterolemic diet and the BGE

306 group diet was supplemented with the BGE extract for 5 weeks. After this feeding period, the BGE

307 group significantly lowered their TC values more than 1.5 folds the HC2 group levels. The

308 reduction might be due to the lower HDL and LDL levels noticed in the BGE group. The different

309 diet did not modified the other determined parameters since TG, AST, ALT and glucose values

310 were similar in both groups. These results were in line with previous findings where mice fed with

311 lard supplemented with a β-glucan extract obtained from *P. ostreatus* prevented the TC increase

312 induced by a hypercholesolemic diet administrated during 5 weeks.[28] Similar

313 hypocholesterolemic activities were noticed for *A. bisporus, Grifola frondosa, Flammulina

314 velutipes* and *L. edodes* dietary fibers but on normocholesterolemic rats.[29,30] However, other studies

315 where a β-glucan-enriched extract (from *P. ostreatus*) was administrated as indicated for BGE (as

316 palliative compounds) did not significantly reduced TC levels in mice serum.[31] The different

13

317 mushroom species or the administration of β-glucans in higher concentrations than those utilized in

318 the report might be the reason for the higher effectivity of the BGE extract from *L. edodes.*

319 **4. Conclusions**

320   Ergosterol-enriched extracts were successfully obtained from *Lentinula edodes* fruiting bodies

321 by supercritical CO_2 extraction with levels up to almost 53% ergosterol (approx. 59% of total

322 sterols). Extraction yields were almost linearly increasing with extraction time up to 3h but extracts

323 obtained after 5h contained higher sterol concentrations. Extracts collected in the separators showed

324 slightly different composition being those collected in S1 extracts with higher ergosterol and

325 fungisterol content than those collected in S2, however, ergosta7,22-dienol was exclusively found in

326 S2. The SFE extract collected after 5 h in S1 and commercial ergosterol were also microemulsified

327 and tested as cholesterol displacers using an *in vitro* digestion model where the DMM fraction was

328 isolated. Ergosterol and the SFE extract with or without microemulsion succeeded to reduce

329 cholesterol levels within the DMMs generated showing potential as hypocholesterolemic

330 compounds. However, when they were administrated concomitant with a hypercholesterolemic diet

331 to a mice model, no reduction of serum cholesterol levels were noticed compared to control.

332 Perhaps the hypocholesterolemic effect reported for other SFE extracts was only effective when

333 administrated as palliative ingredients. These results indicated that although *in vitro* testing might

334 be encouraged to screen biological activities of many bioactive compounds, *in vivo* studies should

335 also be performed as they might disagree with the *in vitro* results. For instance, the BGE extract

336 obtained from *L. edodes* with low ergosterol content (0.23%) but high β-glucan concentration

337 (33.5%) showed lower ability to displace cholesterol in the *in vitro* model but it was able to

338 successfully reduce cholesterol levels in hypercholesterolemic mice model. Therefore, the BGE

339 extract more than the one obtained with SFE should be further recommended to test their

340 hypocholesterolemic activities in clinical trials before formulation into a novel functional food.

341 **Conflicts of interest**

342 None

Abbreviations: ERG (ergosterol), SFE (supercritical fluid extraction), DMM (dietary mixed micelle), TC (total cholesterol), NC (normal control), HFHD (high fat high hypercholesteromic diet), HC (high cholesterol), BGE (β-glucan-enriched extract), MERG (microemulsified ergosterol), MSFE (microemulsified supercritical fluid extract), TG (triglycerides), AST (aspartate aminotransferase), ALT (alanine aminotransferase), GLC (glucose).

352 **References**

353 1. Sengupta, S., Koley, H., Dutta, S., & Bhowal, J. (2018). Hypocholesterolemic effect of

354 *Spirulina platensis* (SP) fortified functional soy yogurts on diet-induced hypercholesterolemia.

355 *Journal of Functional Foods*, *48*, 54-64.

356 2. Bhat, B., & Bajaj, B.K. (2018). Hypocholesterolemic and bioactive potential of

357 exopolysaccharide from a probiotic *Enterococcus faecium* K1 isolated from *kalarei*. *Bioresource*

358 *Technology*, *254*, 264-267.

359 3. Gil-Ramirez, A., Morales, D., & Soler-Rivas, C. (2018). Molecular actions of

360 hypocholesterolaemic compounds from edible mushrooms. *Food & Function*, *9*, 53-69.

361 4. Gil-Ramirez, A., Ruiz-Rodriguez, A., Marin, F.R., Reglero, G., & Soler-Rivas, C. (2014).

362 Effect of ergosterol-enriched extracts obtained from *Agaricus bisporus* on cholesterol absorption

363 using an *in vitro* digestion model. *Journal of Functional Foods*, *11*, 589-597.

364 5. Betters, J.L., & Yu, L. (2010). NPC1L1 and cholesterol transport. *FEBS Letters*, *584*,

365 1873-3468.

366 6. Berger, A., Jones, P.J., & Abumweis, S.S. (2004). Plant sterols: factors affecting their

367 efficacy and safety as functional food ingredients. *Lipids in Health and Disease*, *3*, 5.

368 7. Yi, C., Zhong, H., Tong, S., Cao, X., Firempong, C.K., Liu, H., Fu, M., Yang, Y., Feng,

369 Y., Zhang, H., Xu, X., & Yu, J. (2012). Enhanced oral bioavailability of a sterol-loaded

370 microemulsion formulation of *Flammulina velutipes*, a potential antitumor drug. *International*

371 *Journal of Nanomedicine*, *7*, 5067-5078.

372 8. Calpe-Berdiel, L., Escola-Gil, J.C., & Blanco-Vaca, F. (2009). New insights into the

373 molecular actions of plant sterols and stanols in colesterol metabolism. *Atherosclerosis*, *203*, 18-31.

374   9. Gil-Ramirez, A., Caz, V., Martin-Hernandez, R., Marin, F.R., Largo, C., Rodriguez-

375 Casado, A., Tabernero, M., Ruiz-Rodriguez, A., Reglero, G., & Soler-Rivas, C. (2016). Modulation

376 of cholesterol-related gene expression by ergosterol and ergosterol-enriched extracts obtained from

377 *Agaricus bisporus*. *European Journal of Nutrition*, *55*, 1041-1057.

378   10. Kaneko, E., Matsuda, M., Yamada, Y., Tachibana, Y., Shimomura, I., & Makishima, M.

379 (2003). Induction of intestinal ATP-binding cassette transporters by a phytosterol-derived liver X

380 receptor agonist. *The Journal of Biological Chemistry*, *278*, 36091-36098.

381   11. Tzavella, E., Hatzimichael, E., Kostara, C., Bairaktari, E., Elisaf, M., & Tsimihodimos,

382 V. (2017). Sitosterolemia: a multifaceted metabolic disorder with important clinical consequences.

383 *Journal of Clinical Lipidology*, *11*, 1095-1100.

384   12. Khan, A.A., Gani, A., Khanday, F.A., & Massodi, F.A. (2018) Biological and

385 pharmaceutical activities of mushroom β-glucan discussed as a potential functional food ingredient.

386 *Bioactive Carbohydrates and Dietary Fibre*, *16*, 1-13.

387   13. Kitzberger, C.S.G., Lomonaco, R.H., Michielin, E.M.Z., Danielski, L., Correia, J., &

388 Ferreira, S.R.S. (2009). Supercritical fluid extraction of shiitake oil: Curve modeling and extract

389 composition. *Journal of Food Engineering*, *90*, 35-43.

390   14. Morales, D., Gil-Ramirez, A., Smiderle, F.R., Piris, A.J., Ruiz-Rodriguez, A., & Soler-

391 Rivas, C. (2017). Vitamin D-enriched extracts obtained from shiitake mushrooms (*Lentinula

392 edodes*) by supercritical fluid extraction and UV-irradiation. *Innovative Food Science and Emerging

393 Technologies*, *41*, 330-336.

394   15. Gil-Ramirez, A., Aldars-Garcia, L., Palanisamy, M., Jiverdeanu, R.M., Ruiz-Rodriguez,

395 A., Marin, F.R., Reglero, G., & Soler-Rivas, C. (2013). Sterol enriched fractions obtained from

396 *Agaricus bisporus* fruiting bodies and by-products by compressed fluid technologies (PLE and

397 SFE). *Innovative Food Science & Emerging Technologies*, *18*, 101-107.

398   16. Mazzutti, S., Ferreira, S.R.S., Riehl, C.A.S., Smania Jr., A., Smania, F.A., & Martinez,

399   J. (2012). Supercritical fluid extraction of *Agaricus brasiliensis*: Antioxidant and antimicrobial

400   activities. *The Journal of Supercritical Fluids*, *70*, 48-56.

401   17. Heleno, S.A., Prieto, M.A., Barros, L., Rodrigues, A., Barreiro, M.F., & Ferreira,

402   I.C.F.R. (2016). Optimization of microwave-assisted extraction of ergosterol from *Agaricus*

403   *bisporus* L. by-products using response surface methodology. *Food and Bioproducts Processing*,

404   *100*, 25-35.

405   18. Wang, Q., Cheng, J., Wang, L., Yan, S., Wang, R., Zhang, H., Shao, H., Yang, X.

406   (2018). Valorization of spent shiitake substrate for recovery of antitumor fungal sterols by

407   ultrasound-assisted extraction. *Journal of Food Biochemistry*, *42*, e12602.

408   19. Lopez-Padilla, A., Ruiz-Rodriguez, A., Reglero, G., & Fornari, T. (2017). Supercritical

409   carbon dioxide extraction of *Calendula officinalis*: Kinetic modeling and scaling up study. *The*

410   *Journal of Supercritical Fluids*, *130*, 292-300.

411   20. Morales, D., Piris, A.J., Ruiz-Rodriguez, A., Prodanov, M., & Soler-Rivas, C. (2018).

412   Extraction of bioactive compounds against cardiovascular diseases from *Lentinula edodes* using a

413   sequential extraction method. *Biotechnology Progress*, *34*, 746-755.

414   21. Morales, D., Smiderle, F.R., Piris, A.J., Soler-Rivas, C., & Prodanov, M. (2019).

415   Production of a β-D-glucan-rich extract from Shiitake mushrooms (*Lentinula edodes*) by an

416   extraction/microfiltration/reverse osmosis (nanofiltration) process. *Innovative Food Science and*

417   *Emerging Technologies*, *51*, 80-90.

418   22. Jasinghe, V.J., & Perera, C.O. (2005). Distribution of ergosterol in different tissues of

419   mushrooms and its effect on the conversion of ergosterol to vitamin D_2 by UV irradiation. *Food*

420   *Chemistry*, *92*, 541-546.

421   23. Teichmann, A., Dutta, P.C., Staffas, A., & Jagerstad, M. (2007). Sterol and vitamin D_2

422 concentrations in cultivated and wild grown mushrooms: Effects of UV irradiation. *LWT-Food*

423 *Science and Technology*, *40*, 815-822.

424   24. Palanisamy, M., Aldars-Garcia, L., Gil-Ramirez, A., Ruiz-Rodriguez, A., Marin, F.R.,

425 Reglero, G., & Soler-Rivas, C. (2014). Pressurized water extraction of β-glucan enriched fractions

426 with bile acids-binding capacities obtained from edible mushrooms. *Biotechnology Progress*, *30*,

427 391-400.

428   25. Zacherl, C., Eisner, P., & Engel, K-H., (2011). *In vitro* model to correlate viscosity and

429 bile-acid binding capacity of digested water-soluble and insoluble dietary fibres. *Food Chemistry*,

430 *126*, 423-428.

431   26. Tong, L-T., Zhong, K., Liu, L., Zhou, X., Qiu, J., & Zhou, S. (2015). Effect of dietary

432 hull-less barley β-glucan on the cholesterol metabolism of hypercholesterolemic hamsters. *Food*

433 *Chemistry*, *169*, 344-349.

434   27. He, W-S., Cui, D., Li, L., Tong, L-T., Rui, J., Li, H., Zhang, H., & Liu, X. (2019).

435 Cholesterol-reducing effect of ergosterol is modulated via inhibition of cholesterol absorption and

436 promotion of cholesterol excretion. *Journal of Functional Foods*, *57*, 488-496.

437   28. Caz, V., Gil-Ramirez, A., Santamaria, M., Tabernero, M., Soler-Rivas, C., Martin-

438 Hernandez, R., Marin, F.R., Reglero, G., & Largo, C. (2016). Plasma cholesterol-lowering activity

439 of lard functionalized with mushroom extracts is independent of Niemann-Pick C1-like 1 protein

440 and ABC sterol transporter gene expression in hypercholesterolemic mice. *Journal of Agricultural*

441 *and Food Chemistry*, *64*, 1686-1694.

442   29. Fukushima, M., Nakano, M., Morii, Y., Ohashi, T., Fujiwara, Y., & Sonoyama, K.

443 (2000). Hepatic LDL receptor mRNA in rats is increased by dietary mushroom (*Agaricus bisporus*)

444 fiber and sugar beet fiber. *The Journal of Nutrition*, *130*, 2151-2156.

445   30. Fukushima, M., Ohashi, T., Fujiwara, K., Sonoyama, K., & Nakano, M. (2001).

446   Cholesterol-lowering effects of maitake (*Grifola frondosa*) fiber, shiitake (*Lentinus edodes*) fiber,

447   and enokitake (*Flammulina velutipes*) fiber in rats. *Experimental Biology and Medicine*, 226, 758-

448   765.

449   31. Caz, V., Gil-Ramirez, A., Largo, C., Tabernero, M., Santamaria, M., Martin-Hernandez,

450   R., Marin, F.R., Reglero, G., & Soler-Rivas, C. (2015). Modulation of cholesterol-related gene

451   expression by dietary fiber fractions from edible mushrooms. *Journal of Agricultural and Food*

452   *Chemistry*, 63: 7371-7380.

Tables & Figures

Table captions

Table 1. Fungal sterols content in SFE extracts recovered in separator 1 (S1) and separator 2 (S2) at different extraction times (1, 2, 3 and 5 h). n.d. = not detected; [a-c]Different letters denote significant differences ($P < 0.05$) between different extraction times for the same sterol.

Table 2. Total (TC), HDL (HDL) and LDL-cholesterol (LDL), triglycerides (TG), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and glucose (GLC) values of mice fed 9 weeks HFHD (HC2) and HFHD supplemented with BGE from week 4 to 9. Blood samples were collected after 0, 3 and 9 weeks of administration. Asterisks indicate statistically significant differences between control and BGE group (*P<0.05; **P<0.001).

Figures captions

Figure 1. SFE yields obtained in separator 1 (S1), separator 2 (S2) and total (S1+S2) at different extraction times. [a-d]Different letters denote significant differences ($P < 0.05$) between different extraction times.

Figure 2. Cholesterol and ergosterol concentrations in the isolated DMM fractions generated after *in vitro* digestion of lard supplemented with cholesterol (CH) and cholesterol together with ergosterol (ERG), SFE extract (SFE), microemulsified ergosterol (MERG), microemulsified SFE extract (MSFE), ergosterol+β-glucan-enriched extract (ERG+BGE) and only β-glucan-enriched extract (BGE). [a-c]Different letters denote significant differences ($P < 0.05$) between different samples for the same compound.
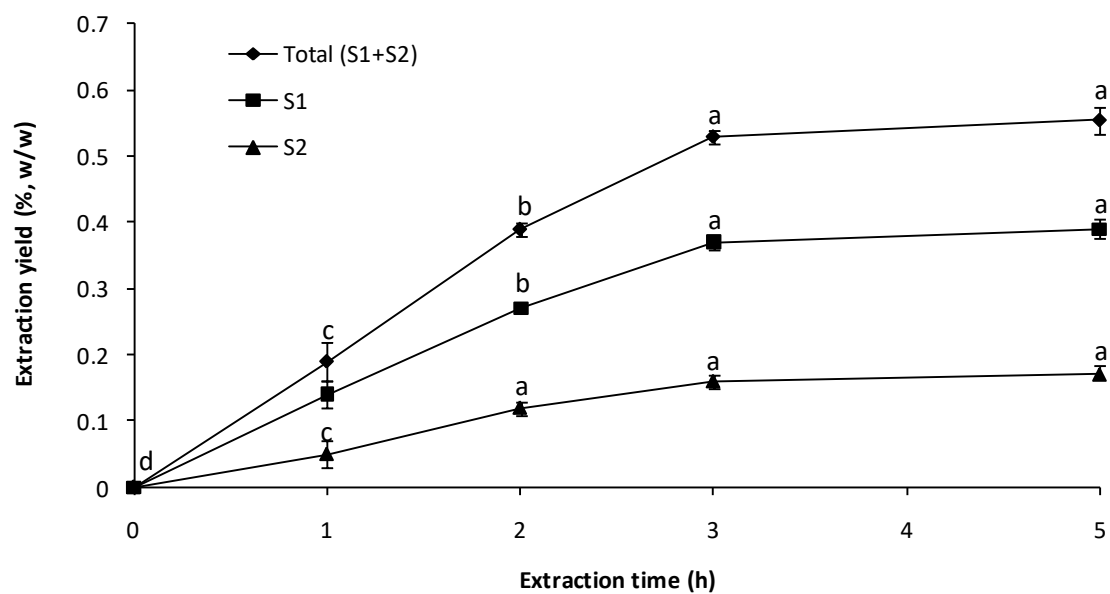
473   **Table 1**

Extraction time (h)	Ergosterol (%)		Ergosta7,22-dienol (%)		Ergosta5,7-dienol (%)		Fungisterol (%)	
	S1	S2	S1	S2	S1	S2	S1	S2
1	41.44±3.40[b]	21.52±2.31[c]	n.d.[b]	1.19±0.28[a]	0.61±0.13[ab]	0.22±0.05[b]	4.15±0.19[b]	2.31±0.23[c]
2	46.30±1.49[ab]	20.30±0.33[c]	n.d.[b]	1.04±0.23[a]	0.48±0.02[ab]	0.16±0.03[b]	3.83±0.05[bc]	1.83±0.45[c]
3	46.12±2.06[ab]	18.56±1.03[c]	n.d.[b]	1.08±0.19[a]	0.46±0.07[ab]	0.40±0.11[b]	3.92±0.13[bc]	2.76±0.49[c]
5	52.50±1.76[a]	25.90±0.18[c]	n.d.[b]	1.54±0.07[a]	0.71±0.09[a]	0.65±0.02[ab]	5.50±0.36[a]	3.79±0.09[bc]

474   **Table 2**

Time (weeks)	Group	TC (mg/dL)	HDL (mmol/L)	LDL (mmol/L)	TG (mg/dL)	AST (UI/dL)	ALT (UI/dL)	GLC (mg/dL)
0	HC2	43.05±8.96	1.01±0.17	0.15±0.03	69.26±19.67	44.49±17.19	10.01±2.47	29.78±12.12
	BGE	34.53±14.35	0.81±0.24*	1.51±2.89	54.83±23.95	47.39±28.26	8.53±4.71	20.43±9.50
3	HC2	96.68±32.72	1.18±0.22	2.06±0.77	30.03±8.12	113.89±57.61	122.56±120.53	56.08±13.98
	BGE	97.11±33.03	1.17±0.22	2.22±0.84	24.78±9.92	71.26±47.39	95.71±130.31	66.78±17.65
9	HC2	259.58±48.26	2.01±0.13	5.60±1.45	34.68±5.66	119.41±61.69	92.61±60.94	64.69±18.57
	BGE	162.51±33.78**	1.62±0.31*	3.62±0.52*	29.97±3.98	154.61±62.64	146.53±74.06	74.70±27.24

Figure 1

Figure 2