



Universidad Autónoma  
de Madrid

**Biblos-e Archivo**  
Repositorio Institucional UAM

Repositorio Institucional de la Universidad Autónoma de Madrid

<https://repositorio.uam.es>

Esta es la **versión de autor** del artículo publicado en:  
This is an **author produced version** of a paper published in:

**Food and Function 10.11 (2019): 7325-7332**

**DOI:** <https://doi.org/10.1039/c9fo01744e>

**Copyright:** © 2019 The Royal Society of Chemistry

El acceso a la versión del editor puede requerir la suscripción del recurso

Access to the published version may require subscription

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

Diego Morales<sup>\*1</sup>, Eva Tejedor-Calvo<sup>1</sup>, Noelia Jurado-Chivato<sup>1</sup>, Gonzalo Polo<sup>2</sup>, María Tabernero<sup>2,3</sup>, Alejandro Ruiz-Rodríguez<sup>1</sup>, Carlota Largo<sup>2</sup>, Cristina Soler-Rivas<sup>1</sup>.

<sup>1</sup>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.

<sup>2</sup>Department of Experimental Surgery, Research Institute Hospital La Paz (IdiPAZ), Paseo de la Castellana 261, 28046 Madrid, Spain

<sup>3</sup>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](mailto:diego.morales@uam.es)

**Keywords:** Shiitake mushrooms; supercritical fluid extraction; ergosterol;  $\beta$ -glucans; cholesterol; *in vitro* digestion.

## 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 °C, 225 bar, 1-5h, 20 L/h recirculated CO<sub>2</sub>).  
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  $\beta$ -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  $\beta$ -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.<sup>1,2</sup>

Edible mushrooms were pointed as one of those foods with potential hypocholesterolemic activities.<sup>3</sup> They contain fungal sterols that (as noticed for phytosterols) might displace cholesterol from dietary mixed micelles (DMMs).<sup>4</sup> 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%).<sup>5,6</sup>

The bioavailability of fungal sterols such as ergosterol could be enhanced by loading it into specific microemulsions improving, for instance, its anti-tumor properties.<sup>7</sup> 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.<sup>8-10</sup> 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.<sup>11</sup> 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  $\beta$ -glucan extracts into ergosterol supplemented food matrices seemed to improve the cholesterol displacement from DMMs.<sup>4</sup> 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,  $\beta$ -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.<sup>12</sup>

Ergosterol-enriched extracts can be successfully obtained from natural sources such as mushrooms using supercritical CO<sub>2</sub> extraction, e.g. from *Lentinula edodes*<sup>13,14</sup>, *Agaricus bisporus*<sup>15</sup> or *Agaricus brasiliensis*.<sup>16</sup> 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.<sup>15,17,18</sup> 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.

In this work, a large-scale SFE plant was used to obtain enough amounts of ergosterol-enriched extract from *Lentinula edodes* to carry out *in vitro* and *in vivo* tests to study its hypocholesterolemic activities. The obtained extract was loaded into a microemulsion and mixed with a fungal  $\beta$ -glucan-enriched extract to investigate whether they might enhance or interfere with its activity as cholesterol displacer from DMMs during an *in vitro* digestion model. Two different mice models were also utilized to evaluate the *in vivo* hypocholesterolemic effect of the SFE extract as well as the  $\beta$ -glucan-enriched extract.

## 2. Materials & Methods

### 2.1. Biological material, standards and reagents

Powdered *Lentinula edodes* S. (Berkeley) fruiting bodies (particle size < 0.5 mm, moisture < 5%) were purchased from Glucanfeed S.L. (La Rioja, Spain) and stored in darkness at -20 °C until further use. Commercially available lard (Iberian pork lard, 99.7 g /100 g fat) was purchased from a local supermarket and maintained at 4 °C until use. All the experiments were performed from the same lot.

Solvents as hexane (95%), chloroform (HPLC grade), methanol (HPLC grade) were obtained from LAB-SCAN (Gliwice, Poland) and absolute ethanol from Panreac (Barcelona, Spain). Calcium chloride, hydrochloric acid (37%), pepsin (from porcine pancreatic mucosa), sodium hydroxide, sodium chloride, Trizma base, maleic acid, pancreatin (from porcine pancreas), L- $\alpha$ -phosphatidyl choline (lecithin), Sepharose® 4B, phenol, ascorbic acid, BHT (2,6-Di-tert-butyl-*p*-cresol) and Kolliphor®EL were purchased from Sigma-Aldrich Química (Madrid, Spain) as well as cholesterol (96%) and ergosterol (95%) used as standards. Carbon dioxide (99.99% purity) was supplied by Air-Liquid España, S.A. (Madrid, Spain).

### 2.2. Supercritical fluid extractions (SFE)

Extractions with CO<sub>2</sub> were carried out in a large scale SFE plant (Zean Consultores S.L., Madrid, Spain) comprising a 6 L cylinder extraction vessel and two different separators (S1 and S2) of 1.6 L capacity each with independent control of temperature and pressure. A detailed explanation of the experimental device can be found elsewhere.<sup>19</sup> The CO<sub>2</sub> flow was set at 20 L/h and during the

total extraction time tested (1 to 5 h) it was recirculated. The temperature was fixed at 40 °C in the extraction vessel and the separators and the extraction pressure was maintained at 225 bar. Ethanol was not used as cosolvent because previous studies indicated that although higher yields could be obtained, the use of only CO<sub>2</sub> was more selective generating fractions with higher sterols concentrations<sup>15</sup>. The fractions extracted in 2 separators (S1 and S2) were collected with ethanol and immediately submitted to dryness on a rotary vacuum evaporator. Ethanol was utilized because it was used in previous studies<sup>19</sup> since it is an organic solvent easy to evaporate with mild processes and it was able to completely solubilize separated fractions detaching the extracts from separator walls. The dried extracts were stored at -20 °C until further analysis.

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

Sterol-loaded microemulsions were formulated as indicated by Yi et al. (2012)<sup>7</sup> with slight modifications. Briefly, ergosterol (50 mg) or a selected SFE extract containing 52.5% ergosterol (SFE) (95 mg) were mixed with lard (1 g). Afterwards, Koliphor EL (21% w/w) and 5% ethanol were mixed thoroughly and stirred as surfactant-cosurfactant solution and mixed with 3% of the supplemented lards. Then, water (71%) was added dropwise into the oily mixtures with gentle stirring to generate microemulsions. Two different microemulsions, MERG (microemulsified ergosterol) and MSFE (microemulsified SFE extract) were prepared and immediately submitted to the *in vitro* digestion model.

### **2.4. Preparation of a $\beta$ -glucan-enriched (BGE) extract**

A  $\beta$ -glucan-enriched extract (BGE) was prepared by mixing different polysaccharides fractions with hypocholesterolemic activities such as a fraction (2.5%) containing water soluble  $\beta$ -glucans (named ExA by Morales et al. (2018)<sup>20</sup>, a fraction (26%) extracted with hot water (98 °C), filtered through a multichannel ceramic membrane (Ceramem Corporation, Waltham, USA) and concentrated with a spiral wound Nanomax50 membrane (Millipore, Bedford, USA) (called RF2 in Morales et al. (2019)<sup>21</sup>) and the remaining fraction (71.5%) containing mainly chitins and insoluble  $\beta$ -glucans.<sup>21</sup> The fractions were lyophilized, pooled together and stored at -20°C until further use.

The resulting mixture contained 33.5% (w/w dry weight)  $\beta$ -glucans and 0.23% ergosterol (determined as Morales et al. (2018)<sup>20</sup>).

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

Lard supplemented with cholesterol (25 mg/g) was used as food matrix to evaluate the hypocholesterolemic activity of the different extracts within an *in vitro* digestion model as indicated by Gil-Ramirez et al. (2014)<sup>4</sup>. The hypercholesterolemic lard was mixed with ergosterol (ERG) (50 mg/g), MERG (32.3 g/g), SFE (95 mg/g) or MSFE (32.3 g/g) as ergosterol-containing formulations and/or BGE extract (149 mg/g) as  $\beta$ -glucan plus ergosterol-containing extract (the indicated concentrations were adjusted to include 50 mg/g ergosterol and/or  $\beta$ -glucans in the food matrix). The mixtures were gently stirred at their melting temperature until complete incorporation of the supplements into the lipidic matrices.

The supplemented food matrices (1 g) were submitted to *in vitro* digestion following the procedure described by Gil-Ramirez et al. (2014)<sup>4</sup>. Afterwards, the fraction containing the dietary mixed micelles (DMM) was isolated using a Sepharose®4B column with 0.15M NaCl/16 mM bile salts as mobile phase as indicated by their intermicellar bile salt concentration (IMBC). The DMM fractions (16 mL) were identified because their cholesterol concentration (determined by an enzymatic SpinReact cholesterol quantification kit (SpinReact SAU, Girona, Spain) co-eluted with their phospholipids concentration (determined using an enzymatic Wako kit (Wako, Madrid, Spain)) at the proper elution volume (Gil-Ramirez et al., 2014)<sup>4</sup>.

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

Sterols were extracted from the samples and quantified following the procedure described by Gil-Ramirez et al. (2013).<sup>15</sup> The unsaponified fractions were injected into an Agilent 19091S-433 capillary column (30 m x 0.25 mm ID and 0.25  $\mu$ m phase thickness). The column was connected to a 7890A System gas chromatograph (Agilent Technologies, Santa Clara, USA) including a G4513A auto injector and a 5975C triple-axis mass spectrometer detector. The injector and detector conditions, as well as the column temperature program, were those described by Gil-



Ramirez et al. (2013).<sup>15</sup> Cholesterol and ergosterol were used as standards and hexadecane (10% v/v) as internal standard.

GC-MS database identified the obtained peaks in concordance with previous studies.<sup>15,22,23</sup> The major detected sterols were cholesterol (RT = 11.9 min), ergosterol (ergosta-5,7,22-trien-3 $\beta$ -ol) (RT= 12.6 min), ergosta 7,22-dienol (RT =12.8 min), ergosta-5,7-dienol (RT = 13.1 min) and ergosta-7-enol (fungisterol) (RT= 13.3 min).

## **2.7. Animal trials**

Male mice C57/BL6JRj (6 weeks; Janvier SAS, Le Genest-Saint-Isle, France) were maintained in temperature-, humidity- and light-controlled conditions (24 $\pm$ 2°C, 40-60% humidity, 12:12 hour light: dark cycle) and had free access to water and food. Two different experiments were carried out and both were approved by the Institutional Animal Welfare and Ethics Committee of La Paz University Hospital (Madrid, Spain) according to the current Spanish and European legislation (RD53/2013 and EU 63/2010).

In the first experiment, 35 mice were randomly divided in four groups. Group NC (normal control, n = 5) was fed a control diet (standard diet, Safe Rodent Diet, A04, Augy, France), group HC1 (high cholesterol, n = 10) was administrated a high fat hypercholesterolemic diet (HFHD) (standard diet supplemented with 1.25% cholesterol, 0.5% cholic acid and 12% lard), group ERG (n = 10) was given the HFHD supplemented with ergosterol (0.45%) and group SFE (n = 10) was fed HFHD supplemented with SFE extract (up to 0.45% ergosterol in the diet). Blood samples were obtained by mandibular puncture at the beginning and the end of the study (5 weeks) and plasma samples were stored at -20 °C until further use.

In the second experiment, 20 mice were fed the standard diet supplemented with 1.25% cholesterol and 0.5% cholic acid and the blood samples were obtained at the beginning and 3 weeks after the feeding started. After 4 weeks, animals were randomly divided in two groups (n = 10), one continued with the same diet and it was used as control (HC2) and the other (BGE) was administrated the diet supplemented with 6% BGE. Both were fed during 5 weeks.

After the feeding periods, mice from both experiments were killed by intracardiac exsanguination under anesthesia with 1.5 % isoflurane, and plasma was collected and stored at  $-20^{\circ}\text{C}$  before use. Liver, fat, kidney and samples were removed and immediately frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ . Plasma levels of total cholesterol, triglycerides, HDL-cholesterol and LDL-cholesterol were measured using a Covas C311 Autoanalyzer (Roche, Spain).

## 2.9. Statistical analysis

Differences were evaluated at 95% confidence level ( $P \leq 0.05$ ) using one-way analysis of variance (ANOVA) followed by Tukey's Multiple Comparison test. Statistical analysis was performed using SPSS V.13.0 software (SPSS Institute Inc., Cary, NC) and GraphPad Prism version 5.01 (GraphPad Software, San Diego, CA, USA).

## 3. Results and discussion

### 3.1 Upscaling of SFE extractions

Large-scale supercritical  $\text{CO}_2$  extractions were carried out to scale up the production of SFE extracts from shiitake mushrooms since similar extracts obtained from *Agaricus bisporus* decreased the TC/HDL ratio in hypercholesterolemic mice when applied at low concentrations. In this sense, Gil-Ramirez et al. (2016)<sup>9</sup> concluded that, in order to improve its hypocholesterolemic activity, higher concentrations of SFE extracts should be utilized for *in vivo* testing.

Extraction yields were increasing with extraction time up to 3h reaching values of 0.53% taking into consideration the material collected in both separators (S1+S2) (Figure 1). However, when longer extraction times were tested (up to 5h) only a slight increase was noticed (0.56 % yield) after 2h more of extraction. The S1 separator collected more material than S2 (2.3-2.8 folds) independently of the extraction time. Previous works carrying out SFE extractions from shiitake mushrooms with a pilot scale plant and similar conditions (225 bar,  $35^{\circ}\text{C}$ , 3 h) showed higher yields (1.25%).<sup>14</sup> Those yields could be improved in pilot-plants by increasing temperature (2.3% at 225 bar and  $75^{\circ}\text{C}$ ) or pressure (2.1% at 350 bar and  $40^{\circ}\text{C}$ ). Nevertheless, other authors obtained similar

values in shiitake SFE at 40 °C during 3 h under 200 and 250 bar (0.65 and 0.81%, respectively) using smaller plant with a 100 mL extraction cell.<sup>13</sup>

The obtained SFE extracts contained up to 53% ergosterol (Table 1), similar values than those indicated by Gil-Ramirez et al. (2013)<sup>15</sup> for *Agaricus bisporus* but significantly higher than others noticed for *L. edodes* using a smaller capacity pilot plant.<sup>14</sup> Results showed that, although the yield in this large-scale plant was lower, the extraction was apparently more selective toward sterols than when using a pilot plant. Moreover, when the ergosterol content was determined in the extracts collected in both separators, the S1 extracts exhibited higher cholesterol concentrations (41-53%) than S2 extracts (19-26%) and statistically significant differences were found in S1 between 1 and 5 h of extraction time suggesting that time could influence ergosterol enrichment of the extracts.

Ergosterol derivatives were also detected in the extracts, although at lower concentrations (Table 1). Fungisterol concentrations were higher than ergosta5,7-dienol and ergosta7,22-dienol and it was distributed within the separators in a similar ratio to ergosterol. However, ergosta7,22-dienol was specifically recovered in S2 and ergosta5,7-dienol values showed no differences between separators. Thus, the extract containing the larger amount of fungal sterols (58.7%) obtained after 5h in S1 was selected as SFE extract to carry out further experiments.

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

Previous *in vitro* studies indicated that ergosterol and particularly a SFE extract containing fungal sterols from *A. bisporus*, were as effective as  $\beta$ -sitosterol in the displacement of cholesterol from DMM when incorporated in a food matrix such as lard.<sup>4</sup> In order to test the ability of the SFE extract obtained from *L. edodes*, the same *in vitro* digestion model was reproduced. Results indicated that the DMM fraction generated after digestion of lard supplemented with cholesterol and ergosterol (ERG) contained approx. 63% less cholesterol than when only cholesterol was added (Figure 2). This reduction was slightly higher than the 49% noticed by Gil-Ramirez et al. (2014)<sup>4</sup> but the effect reported when the SFE extracts were added, was almost the same (69% and 67%, respectively) indicating that, independently of the mushroom utilized, the SFE extracts were able to

reduce cholesterol levels in the DMM fraction (that is the one usually absorbed by intestinal enterocytes).

Moreover, according to previous studies, the bioavailability of phytosterols, as well as other non-cholesterol sterols was very limited<sup>8</sup> but other reports indicated that ergosterol bioavailability might be enhanced by loading them into microemulsions.<sup>7</sup> However, if sterols are incorporated into artificial vesicles and these are incorporated into food matrices, they might be more bioavailable but perhaps they are not able to act as cholesterol displacers in the DMMs. Therefore, ergosterol and the SFE extract were emulsified (MERG and MSFE, respectively), supplemented to the hypercholesterolemic lard and digested to investigate the content of the DMM fractions generated. Results indicated that both extracts could displace cholesterol from the generated DMMs as their corresponding non-microemulsified extracts.

Other reports suggested that the presence of  $\beta$ -glucans extracts obtained from *Pleurotus ostreatus* in the food matrix functionalized with ergosterol reduced the cholesterol content of the generated DMMs approx. 52% more than if only the sterol was added.<sup>4</sup> When similar food mixture was prepared but using a  $\beta$ -glucan-enriched extract obtained from *L. edodes* (BGE+ERG), a lower cholesterol level was noticed in the DMM fraction generated than in the DMM control (CH), however, no significantly different values were noted compared to the DMM fraction generated when the food matrix was supplemented only with ERG. Differences with previous investigations might be due to the structural differences between the polysaccharides from both mushroom species or because of their specific extract compositions. Surprisingly, when the BGE extract was also tested without any sterol addition, significant cholesterol reduction was also noticed (33.7%) in its DMM fraction compared to the control. Since the ergosterol levels in BGE extract were really low (0.23%), the noticed hypocholesterolemic activity might be because of its high  $\beta$ -glucans content (33.5%). These polymers were able to scavenge other small molecules such as bile acids or cholesterol in their structures.<sup>24,25</sup> In fact, it was suggested as their potential mechanism to lower cholesterol levels in serum<sup>12</sup> although there might be others.<sup>26</sup>

Ergosterol was also detected in the generated DMM fractions but it was always incorporated in lower concentrations than cholesterol (on average 58% less) corroborating previous results.<sup>4</sup> Moreover, no significant differences were noticed between the different tested extracts suggesting that its incorporation in the DMM was more related to the ergosterol content of the food matrix than to the presence of other components in the mixture (such as *i.e.*  $\beta$ -glucans, surfactants, etc.) because all the extracts were supplemented to the hypercholesterolemic lard in different concentrations to ensure the same ergosterol content.

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

Animal studies were carried out to confirm the hypocholesterolemic activity noticed *in vitro* for the extracts. However, in order to minimize the number of mice, only ergosterol as standard compound and the SFE extract were tested since no significant differences were found with the MERG or MSFE in their capacity to displace cholesterol from DMMs. The MSFE preparation included more processing that might increase the cost if up scaled. Moreover, the administration of the preparations was carried out at the same time than the induction of hypercholesterolemia to study their potential as preventive more than palliative formulations since the latter influence was already investigated for a SFE extract obtained from *A. bisporus*.<sup>9</sup>

The mice groups treated with normal diet (NC) or hypercholesterolemic diet (HC1) used as control as well as the ERG and SFE groups showed at the beginning of the experimental period similar values of total cholesterol (TC) (on average  $91.4 \pm 10.8$  mg/dL), HDL ( $1.9 \pm 0.7$  mmol/L), LDL ( $0.2 \pm 0.1$  mmol/L), triglycerides (TG) ( $158 \pm 68.8$  mg/dL), AST ( $74.8 \pm 36.1$  UI/dL), ALT ( $18.0 \pm 8.1$  UI/dL) and glucose  $50.0 \pm 27.5$  (mg/dL). However, after 5 weeks only the NC group maintained those levels and the rest of groups increased their TC levels with non-significant differences between HC1 ( $230.3 \pm 67.1$  mg/dL), ERG ( $277.3 \pm 35.5$  mg/dL) or SFE ( $262 \pm 52.6$  mg/dL) groups. The increase noticed in ERG group was more influenced by a high LDL value ( $5.7 \pm 1.0$  while HC1 showed  $4.6 \pm 1.7$  mmol/L) and the one noticed in the SFE group by a high HDL value ( $3.2 \pm 1.0$  while HC1 showed  $2.2 \pm 0.3$  mmol/L), although differences in both parameters were also

insignificant. Similarly, the TC and glucose values increased in all the groups (40.5 to 45.6 mg/dL and 155.5 to 192.7 mg/dL, respectively). Therefore, the ergosterol containing preparations showed no hypocholesterolemic activity *in vivo*. These results differed from recent studies carried out in rats fed simultaneously a hypercholesterolemic diet supplemented with 0.5% or 1.5% ergosterol for 8 weeks<sup>27</sup> or previous tests carried out in mice but where firstly hypercholesterolemia was induced and then treated with ergosterol-containing preparations obtained from *A. bisporus*.<sup>9</sup>

Thus, since the BGE extracts also showed hypocholesterolemic activity *in vitro* but their mechanism of action might be different than fungal sterols, another *in vivo* experiment was carried out to investigate whether the  $\beta$ -glucan-enriched extract was more effective than fungal sterols. This time, the experimental setting was changed and firstly, mice were fed a hypercholesterolemic diet and then they were administrated the BGE extract together with the unhealthy diet to investigate their effect as palliative compounds. Results indicated that administration of the standard diet supplemented with cholesterol and cholic acid for 3 weeks increased TC, HDL and LDL levels in all the mice inducing hypercholesterolemia (Table 2). The glucose concentration and hepatic enzymes activities were also influenced and increased their values remaining only the TG amounts unchanged. After 4 weeks, HC2 group continued with the hypercholesterolemic diet and the BGE group diet was supplemented with the BGE extract for 5 weeks. After this feeding period, the BGE group significantly lowered their TC values more than 1.5 folds the HC2 group levels. The reduction might be due to the lower HDL and LDL levels noticed in the BGE group. The different diet did not modified the other determined parameters since TG, AST, ALT and glucose values were similar in both groups. These results were in line with previous findings where mice fed with lard supplemented with a  $\beta$ -glucan extract obtained from *P. ostreatus* prevented the TC increase induced by a hypercholesterolemic diet administrated during 5 weeks.<sup>28</sup> Similar hypocholesterolemic activities were noticed for *A. bisporus*, *Grifola frondosa*, *Flammulina velutipes* and *L. edodes* dietary fibers but on normocholesterolemic rats.<sup>29,30</sup> However, other studies where a  $\beta$ -glucan-enriched extract (from *P. ostreatus*) was administrated as indicated for BGE (as palliative compounds) did not significantly reduced TC levels in mice serum.<sup>31</sup> The different

mushroom species or the administration of  $\beta$ -glucans in higher concentrations than those utilized in the report might be the reason for the higher effectivity of the BGE extract from *L. edodes*.

#### 4. Conclusions

Ergosterol-enriched extracts were successfully obtained from *Lentinula edodes* fruiting bodies by supercritical CO<sub>2</sub> extraction with levels up to almost 53% ergosterol (approx. 59% of total sterols). Extraction yields were almost linearly increasing with extraction time up to 3h but extracts obtained after 5h contained higher sterol concentrations. Extracts collected in the separators showed slightly different composition being those collected in S1 extracts with higher ergosterol and fungisterol content than those collected in S2, however, ergosta7,22-dienol was exclusively found in S2. The SFE extract collected after 5 h in S1 and commercial ergosterol were also microemulsified and tested as cholesterol displacers using an *in vitro* digestion model where the DMM fraction was isolated. Ergosterol and the SFE extract with or without microemulsion succeeded to reduce cholesterol levels within the DMMs generated showing potential as hypocholesterolemic compounds. However, when they were administrated concomitant with a hypercholesterolemic diet to a mice model, no reduction of serum cholesterol levels were noticed compared to control. Perhaps the hypocholesterolemic effect reported for other SFE extracts was only effective when administrated as palliative ingredients. These results indicated that although *in vitro* testing might be encouraged to screen biological activities of many bioactive compounds, *in vivo* studies should also be performed as they might disagree with the *in vitro* results. For instance, the BGE extract obtained from *L. edodes* with low ergosterol content (0.23%) but high  $\beta$ -glucan concentration (33.5%) showed lower ability to displace cholesterol in the *in vitro* model but it was able to successfully reduce cholesterol levels in hypercholesterolemic mice model. Therefore, the BGE extract more than the one obtained with SFE should be further recommended to test their hypocholesterolemic activities in clinical trials before formulation into a novel functional food.

#### Conflicts of interest

None

343    **Acknowledgements**

344    This research was supported by national R+D program from the Spanish Ministry of Science and  
345    Innovation (project AGL2014-26211-R) and the regional program from the Community of Madrid,  
346    Spain (S2013/ABI-2728).

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



## References

1. Sengupta, S., Koley, H., Dutta, S., & Bhowal, J. (2018). Hypocholesterolemic effect of *Spirulina platensis* (SP) fortified functional soy yogurts on diet-induced hypercholesterolemia. *Journal of Functional Foods*, 48, 54-64.
2. Bhat, B., & Bajaj, B.K. (2018). Hypocholesterolemic and bioactive potential of exopolysaccharide from a probiotic *Enterococcus faecium* K1 isolated from *kalarei*. *Bioresource Technology*, 254, 264-267.
3. Gil-Ramirez, A., Morales, D., & Soler-Rivas, C. (2018). Molecular actions of hypocholesterolaemic compounds from edible mushrooms. *Food & Function*, 9, 53-69.
4. Gil-Ramirez, A., Ruiz-Rodriguez, A., Marin, F.R., Reglero, G., & Soler-Rivas, C. (2014). Effect of ergosterol-enriched extracts obtained from *Agaricus bisporus* on cholesterol absorption using an *in vitro* digestion model. *Journal of Functional Foods*, 11, 589-597.
5. Betters, J.L., & Yu, L. (2010). NPC1L1 and cholesterol transport. *FEBS Letters*, 584, 1873-3468.
6. Berger, A., Jones, P.J., & Abumweis, S.S. (2004). Plant sterols: factors affecting their efficacy and safety as functional food ingredients. *Lipids in Health and Disease*, 3, 5.
7. Yi, C., Zhong, H., Tong, S., Cao, X., Firempong, C.K., Liu, H., Fu, M., Yang, Y., Feng, Y., Zhang, H., Xu, X., & Yu, J. (2012). Enhanced oral bioavailability of a sterol-loaded microemulsion formulation of *Flammulina velutipes*, a potential antitumor drug. *International Journal of Nanomedicine*, 7, 5067-5078.
8. Calpe-Berdiel, L., Escola-Gil, J.C., & Blanco-Vaca, F. (2009). New insights into the molecular actions of plant sterols and stanols in cholesterol metabolism. *Atherosclerosis*, 203, 18-31.

9. Gil-Ramirez, A., Caz, V., Martin-Hernandez, R., Marin, F.R., Largo, C., Rodriguez-Casado, A., Tabernero, M., Ruiz-Rodriguez, A., Reglero, G., & Soler-Rivas, C. (2016). Modulation of cholesterol-related gene expression by ergosterol and ergosterol-enriched extracts obtained from *Agaricus bisporus*. *European Journal of Nutrition*, 55, 1041-1057.
10. Kaneko, E., Matsuda, M., Yamada, Y., Tachibana, Y., Shimomura, I., & Makishima, M. (2003). Induction of intestinal ATP-binding cassette transporters by a phytosterol-derived liver X receptor agonist. *The Journal of Biological Chemistry*, 278, 36091-36098.
11. Tzavella, E., Hatzimichael, E., Kostara, C., Bairaktari, E., Elisaf, M., & Tsimihodimos, V. (2017). Sitosterolemia: a multifaceted metabolic disorder with important clinical consequences. *Journal of Clinical Lipidology*, 11, 1095-1100.
12. Khan, A.A., Gani, A., Khanday, F.A., & Massodi, F.A. (2018) Biological and pharmaceutical activities of mushroom  $\beta$ -glucan discussed as a potential functional food ingredient. *Bioactive Carbohydrates and Dietary Fibre*, 16, 1-13.
13. Kitzberger, C.S.G., Lomonaco, R.H., Michielin, E.M.Z., Danielski, L., Correia, J., & Ferreira, S.R.S. (2009). Supercritical fluid extraction of shiitake oil: Curve modeling and extract composition. *Journal of Food Engineering*, 90, 35-43.
14. Morales, D., Gil-Ramirez, A., Smiderle, F.R., Piris, A.J., Ruiz-Rodriguez, A., & Soler-Rivas, C. (2017). Vitamin D-enriched extracts obtained from shiitake mushrooms (*Lentinula edodes*) by supercritical fluid extraction and UV-irradiation. *Innovative Food Science and Emerging Technologies*, 41, 330-336.
15. Gil-Ramirez, A., Aldars-Garcia, L., Palanisamy, M., Jiverdeanu, R.M., Ruiz-Rodriguez, A., Marin, F.R., Reglero, G., & Soler-Rivas, C. (2013). Sterol enriched fractions obtained from *Agaricus bisporus* fruiting bodies and by-products by compressed fluid technologies (PLE and SFE). *Innovative Food Science & Emerging Technologies*, 18, 101-107.

16. Mazzutti, S., Ferreira, S.R.S., Riehl, C.A.S., Smania Jr., A., Smania, F.A., & Martinez, J. (2012). Supercritical fluid extraction of *Agaricus brasiliensis*: Antioxidant and antimicrobial activities. *The Journal of Supercritical Fluids*, 70, 48-56.
17. Heleno, S.A., Prieto, M.A., Barros, L., Rodrigues, A., Barreiro, M.F., & Ferreira, I.C.F.R. (2016). Optimization of microwave-assisted extraction of ergosterol from *Agaricus bisporus* L. by-products using response surface methodology. *Food and Bioprocess Processing*, 100, 25-35.
18. Wang, Q., Cheng, J., Wang, L., Yan, S., Wang, R., Zhang, H., Shao, H., Yang, X. (2018). Valorization of spent shiitake substrate for recovery of antitumor fungal sterols by ultrasound-assisted extraction. *Journal of Food Biochemistry*, 42, e12602.
19. Lopez-Padilla, A., Ruiz-Rodriguez, A., Reglero, G., & Fornari, T. (2017). Supercritical carbon dioxide extraction of *Calendula officinalis*: Kinetic modeling and scaling up study. *The Journal of Supercritical Fluids*, 130, 292-300.
20. Morales, D., Piris, A.J., Ruiz-Rodriguez, A., Prodanov, M., & Soler-Rivas, C. (2018). Extraction of bioactive compounds against cardiovascular diseases from *Lentinula edodes* using a sequential extraction method. *Biotechnology Progress*, 34, 746-755.
21. Morales, D., Smiderle, F.R., Piris, A.J., Soler-Rivas, C., & Prodanov, M. (2019). Production of a  $\beta$ -D-glucan-rich extract from Shiitake mushrooms (*Lentinula edodes*) by an extraction/microfiltration/reverse osmosis (nanofiltration) process. *Innovative Food Science and Emerging Technologies*, 51, 80-90.
22. Jasinghe, V.J., & Perera, C.O. (2005). Distribution of ergosterol in different tissues of mushrooms and its effect on the conversion of ergosterol to vitamin D<sub>2</sub> by UV irradiation. *Food Chemistry*, 92, 541-546.

- 421           23. Teichmann, A., Dutta, P.C., Staffas, A., & Jagerstad, M. (2007). Sterol and vitamin D<sub>2</sub>  
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  $\beta$ -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  $\beta$ -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; <sup>a-c</sup>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. <sup>a-d</sup>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+ $\beta$ -glucan-enriched extract (ERG+BGE) and only  $\beta$ -glucan-enriched extract (BGE). <sup>a-c</sup>Different letters denote significant differences ( $P < 0.05$ ) between different samples for the same compound.

473 **Table 1**

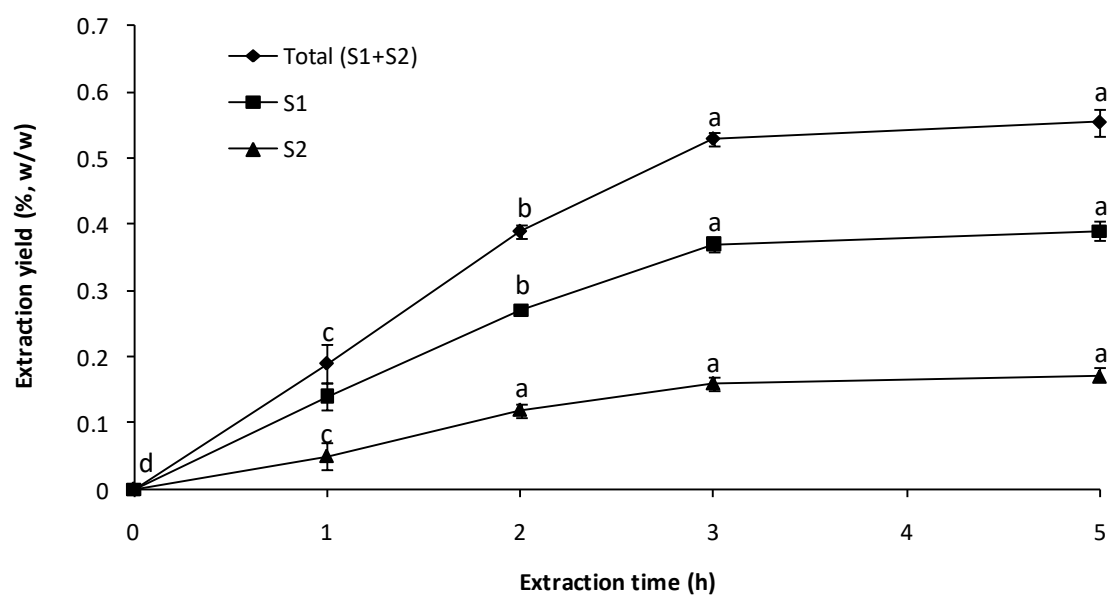
Extraction time (h)	Ergosterol (%)		Ergosta7,22-dienol (%)		Ergosta5,7-dienol (%)		Fungisterol (%)	
	S1	S2	S1	S2	S1	S2	S1	S2
<b>1</b>	41.44±3.40 <sup>b</sup>	21.52±2.31 <sup>c</sup>	n.d. <sup>b</sup>	1.19±0.28 <sup>a</sup>	0.61±0.13 <sup>ab</sup>	0.22±0.05 <sup>b</sup>	4.15±0.19 <sup>b</sup>	2.31±0.23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

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



475 **Figure 1**



476 **Figure 2**

