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Characterization of the Nutritional Composition of a Biotechnologically Produced Oyster Mushroom and its Physiological Effects in Obese Zucker Rats

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Scope: Sustainable protein sources are needed to meet the increasing protein demands of a continuously growing world population. This study is focused on the biotechnological production of a protein rich oyster mushroom (*Pleurotus sajor-caju*; PSC) by valorization of an agricultural side stream and the evaluation of the physiological effects of PSC in a rat model of metabolic syndrome.

Methods and results: PSC is produced via submerged cultivation in a 150 L bioreactor that utilizes isomaltulose molasses as its sole carbon source, and is further analyzed for its nutritional composition. A feeding trial is performed using Zucker rats which are fed a 5% PSC supplemented diet, for 4 weeks. Biochemical analyses reveal a significant reduction of the liver lipid concentrations and liver inflammation in the PSC fed obese rats in comparison to the obese rats from the control group. Hepatic qPCR analyses, differential transcript profiling, and enzyme activity measurements reveal a number of altered pathways that may be responsible for these anti-steatotic and anti-inflammatory effects of the mushroom.

Conclusion: Bioconversion of a low quality agricultural side stream to an improved protein source is performed by submerged cultured PSC, and the obtained mycelium shows strong anti-steatotic and anti-inflammatory effects.

1. Introduction

A growing population, combined with factors like food production from animal origin and agriculture are currently placing an increased pressure on the environment.[1,2] If no mitigation measures are taken, adverse impacts of these factors on environmental change are estimated to increase by 70% until 2050.[2,3] However, food consumption is not only crucial in view of ecosystem health. Over the past decades, dietary patterns have been shifting considerably, from under- to over-nutrition and away from freshly produced to processed food high in carbohydrates, fat, and sugar and to animal-based protein.[2,4] At the intersection of human and ecosystem health, it will be a major challenge to re-align future protein supply and demand that accounts for both nutritional requirements and environmental implications.[2,5] The urgent need to deal with the above-mentioned scenarios collectively, has pushed researchers to look for sustainable alternative protein sources.

Agricultural production and the agro-food industry in most countries furnish large volumes of solid wastes and by-products which have a very high organic load.[6] Alternatively, valorization of low-grade agricultural waste by edible fungi, commonly known as mushrooms, is considered to represent a promising strategy for the production of protein rich biomass since they boast of a unique enzyme system that has the ability to recover nutrients and energy from biodegradable waste. Compositional analyses of cultivated varieties (fruiting bodies) have revealed that, on a dry matter (DM) basis, mushrooms normally contain 19–35% protein. Moreover, mushroom proteins contain all essential amino acids.[7] The low total fat content and the high proportion of polyunsaturated fatty acids (up to 85%) relative to total fatty acids, is considered a significant contributor to the health value of mushrooms.[7] Mushrooms also contain relatively large amounts of carbohydrates and dietary fiber ranging from 51% to 88% and 4 to 20% (DM), respectively.[7]

Apart from being acknowledged for their nutritional and flavor enhancing properties, mushrooms have also been regarded

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as medicinal and functional foods. Many edible mushrooms, including *Grifola frondosa* (maitake), *Hericium erinaceus*, and *Pleurotus ostreatus* are used in traditional folk medicine.[8] Usually regarded as oyster mushrooms, the genus *Pleurotus* (Pleurotaceae, higher Basidiomycetes) is the third most distributed edible mushroom worldwide and has high nutritional value and therapeutic properties.[9] The multidirectional health-promoting effects of mushrooms of the *Pleurotus* genus result from the presence of secondary metabolites, which have been isolated from both oyster mushroom fruiting bodies and mycelia.[10] The bioactive compounds identified in *Pleurotus* include polysaccharides, chitinous substances, amino acids, fatty acid esters, sterols, and polyphenols. These bioactive substances exhibit antiatherosclerotic, anti-inflammatory, hepatoprotective, and antioxidative properties.[11,12]

Current commercial mushroom products are obtained from fruiting bodies of field-cultivated mushrooms, a time-exhausting and labor-intensive process.[13] Submerged cultivation of mushrooms has received increased attention and has significant industrial potential for efficient production of mycelial biomass and metabolites.[13] It offers the advantages of faster production within reduced space and lesser chances of contamination.[13]

The nutritional composition of mushrooms, as well as their content in various bioactive ingredients, vary quantitatively and qualitatively depending on the strain, cultivation conditions, and substrate used.[14,15] Considering the great interest for mushrooms as an alternative protein source, scarce information is available on the nutritional properties of mycelial biomass derived from submerged fermentation of different species and media.[14] In the present study, usage of isomaltulose molasses (ISM, a side stream of the sugar industry) was utilized as the sole carbon source for production of mycelial biomass of *Pleurotus sajor-caju* (PSC) via submerged cultivation. Owing to the abundance of bioactive metabolites for the development of drugs and nutraceuticals in *Pleurotus* species,[8–12] we further speculated that the biotechnologically produced PSC biomass may have health-related effects. To evaluate these effects, a feeding trial utilizing a rat model of liver steatosis, hyperlipidemia, and obesity was carried out for 4 weeks, in which diets were supplemented with 5% PSC. To assess the physiological effects of PSC, analysis of liver lipids, hepatic gene expression levels related to lipid metabolism and inflammation along with differential hepatic transcript profiling and measurement of hepatic lipogenic enzyme activities was applied.

2. Experimental Section

2.1. Submerged Fermentation and Chemical Analyses of *Pleurotus sajor-caju*

2.1.1. Cultivation of Pleurotus sajor-caju

PSC, as mentioned by Ahlborn et al.,[16] was obtained from the culture collection present at the Institute of Food Chemistry and Food Biotechnology, Justus Liebig University, Giessen, Germany.

For the preculture and main culture media, chemicals were obtained from Carl Roth (Karlsruhe, Germany), Sigma-Aldrich (Taufkirchen, Germany), and Th. Geyer (Hamburg, Germany). ISM was obtained from Suedzucker (Offstein, Germany).

PSC was cultivated with ISM according to Trapp et al.[17] The detailed procedure has been described in Section S1, Supporting Information.

A 150 L bioreactor (Biostat D 100, B. Braun International, Melsungen, Germany) was used to produce PSC. The bioreactor was equipped with a Rushton-type impeller, a pH electrode and a temperature sensor. The main culture was grown at 24 °C and 150 rpm with an air-flow rate of 3.0 L min^{-1}. After 6 days, PSC was harvested with a sieve (250 micron; food grade), thoroughly washed with water, freeze died and stored at −20 °C.

2.1.2. Chemical Analyses of Pleurotus sajor-caju

Determination of the crude protein, ash, fat, fatty acid profile, and amino acids in PSC were performed as described by Ahlborn et al.[16] The amount of carbohydrates was calculated as difference to 100%. For quantitation of glucans, the enzyme assay kit—mushroom and yeast beta-glucan from Megazyme Inc. (Ireland) was utilized according to the manufacturer's protocol. Chitin quantitation employed a colorimetric assay with 3-methyl-2-benzothiazolinone hydrazone hydrochloride at 650 nm, with modifications.[16,18] Ergothioneine, a sulphur containing amino acid, was quantified in PSC, as described previously,[19] with modifications. Detailed methods have been provided in Section S2, Supporting Information.

2.2. Animal Study

2.2.1. Animals, Diets, and Experimental Design

The animal experiment was approved by the local Animal Care and Use Committee (Regierungspräsidium Giessen; permission no: JLU 648_M). All experimental procedures followed established guidelines for handling of laboratory animals. This study used 24 male, 5–6-week-old, homozygous (fa/fa) obese Zucker rats (Crl:ZUC-*Lepr*^{fa}) and 24 male, 6–8-week-old, heterozygous (fa/+) lean Zucker rats. Animals were purchased from Charles River (Sulzfeld, Germany). The rats were caged in groups of two animals each and were housed in a temperature-controlled room (22 ± 1 °C ambient temperature, 50–60 % relative humidity) in a 12 h light/dark cycle. The obese rats were randomly assigned to two groups (obese control (OC) and obese 5% PSC (OPSC)) of 12 rats each. Similarly, the lean rats were also randomly assigned to two groups (lean control (LC) and lean 5% PSC (LPSC)) of 12 rats each. The rats were fed two semisynthetic diets with comparable levels of gross energy and crude nutrients, to which they had free access for 4 weeks (**Table 1**). The composition of the diets was selected to equalize concentrations of nutrients including crude protein, crude fat, crude fibre, and crude ash and were sufficient to meet requirements for the maintenance of the rats according to National Research Council (NRC).[20] In groups LC and OC, the semisynthetic diet contained no additional supplementation (control diet), whereas the semisynthetic diet of the groups OPSC and LPSC was first supplemented with 5% PSC and the nutrients were then made up by difference to reach same levels as the control diet. Both diets contained 0.5% titanium dioxide which was used to calculate the apparent total tract digestibility of energy.[21]

Table 1. Composition and nutrient and energy contents of the experimental diets.

	Control diet	5% PSC diet
Components (g kg^{-1})		
Corn starch	550	526.5
Casein	200	192
PSC	-	50
Sucrose	100	100
Soybean oil	50	49
Cellulose	50	32.5
Mineral mix[1)]	35	35
Vitamin mix[2)]	10	10
Titanium dioxide	5	5
Analyzed crude nutrient and energy content		
Dry matter (% FM)	85.2	84.2
Crude protein (% DM)	19.7	20.0
Crude fat (% DM)	5.9	5.9
Crude ash (% DM)	3.1	3.2
Crude fiber (% DM)	3.2	3.0
Gross energy (kcal g^{-1} DM)	4.7	4.7

[1,2)] Detailed composition of the mineral and vitamin mix is available in Section S3, Supporting Information.

Water was available at all times ad libitum from nipple drinkers. Body weight and feed intake were determined weekly in the period of 4 weeks.

2.2.2. Dosage Information

The PSC concentration of 5% was chosen based on recent studies,[12,22–25] in which addition of 5% powdered fruiting bodies of various *Pleurotus* species produced a physiological effect in various rat models of hyperlipidemia. Considering an average body weight of the obese rats of 450 g and an average feed intake of 30 g day^{-1}, the daily dosage of PSC relates to 3.33 g PSC per kg body weight. For a human weighing 70 kg with a daily food intake of 500 g on average, the human equivalent dose (HED) would extrapolate to 0.36 g PSC per kg body weight.

2.2.3. Analysis of Feed Composition

Determination of dry matter, energy content, and concentrations of crude fiber and ash in the diets was performed according to official methods.[26] The fat content, crude protein, and amino acid composition was analyzed as mentioned in Section 2.1.2.

2.2.4. Sample Collection

After 28 days, rats were decapitated under CO_2 anesthesia. Blood was collected into EDTA-coated polyethylene tubes (Sarstedt, Nümbrecht, Germany). Plasma was separated from blood by centrifugation (1100 x *g*; 10 min; 4 °C). The liver was excised and weighed, washed in ice-cold NaCl solution (0.9%) after which several small aliquots were made and snap-frozen in liquid nitrogen. Plasma and liver samples were stored at −80 °C.

2.2.5. Determination of Plasma and/or Liver Lipids

Concentrations of non-esterified fatty acids (NEFA), triacylglycerol (TG), and total cholesterol (CHOL) in the plasma were determined by enzymatic reagent kits, following the instructions of the manufacturer (Code Nos.: 436–91995 and 417–73501, Wako Chemicals GmbH, Neuss, Germany; Fluitest TG, cat. no. 5741; Fluitest CHOL, cat. no. 4241, Analyticon Biotechnologies, Lichtenfels, Germany, respectively). Hepatic lipids were extracted with a mixture of n-hexane and isopropanol (3:2, v/v) as described by Hara and Radin.[27] Lipid extracts were dried and lipids were dissolved with chloroform and Triton X-100 (1:1, v/v).[28] Finally, TG and CHOL contents were measured using the same enzymatic kits as used for the plasma.

2.2.6. Determination of Proinflammatory Cytokines in the Plasma

Concentrations of interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α) in the plasma were determined by commercial enzyme-linked immunosorbent assay kits (ab234570 and ab236712, respectively, Abcam, Cambridge, UK), following the instructions of the manufacturer.

2.2.7. Oil Red O Staining of Liver for Determination of Lipid Accumulation

Lipid accumulation in the liver was evaluated by Oil Red O-staining as described recently.[29] A detailed description is provided in Section S4, Supporting Information.

2.2.8. RNA Extraction and qPCR Analysis

Total RNA from frozen liver aliquots (≈15 mg) was isolated using TRIzol reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer's protocol and subsequently analysed for quantity and quality using an Infinite 200M microplate reader (Tecan, Mainz, Germany). The average RNA concentration and A260/A280 ratio of total RNA from all samples (n = 48) was 0.72 ± 0.27 μg μL^{-1} and 1.96 ± 0.02, respectively. The cDNA was synthesized as described recently.[30] qPCR analysis was carried out using the method described recently.[30] Characteristics of primers used for qPCR analysis are shown in Table S1, Supporting Information. For calculation of mRNA levels, detailed information is present in Section S5, Supporting Information. The mean of OC group was set to 1.0, and the mean and standard deviation of the other groups were scaled proportionally to show the changes in mRNA expression as fold of OC group.

2.2.9. Hepatic GeneChip Microarray Profiling and Data Analysis

For microarray analysis, n = 6 liver total RNA samples/group were randomly selected from the obese groups. After checking RNA quality (A260:A280 ratios and RNA integrity number values (mean ± SD) were 1.90 ± 0.01 and 8.01 ± 0.38, respectively), samples were processed as described,[31] at an Affymetrix

Service Provider and Core Facility, "KFB—Center of Excellence for Fluorescent Bioanalytics" (Regensburg, Germany; www.kfb-regensburg.de). More details can be found in Section S6, Supporting Information. The microarray data have been deposited in MIAME compliant format in the NCBI's Gene Expression Omnibus public repository;[32] GEO accession no. GSE151882. Transcripts were defined as differentially expressed when the fold change (FC) between the OPSC group and OC group was > 1.3, < -1.3 and the p-value of the unpaired Student's t-test was < 0.05. Gene set enrichment analysis (GSEA) was performed in order to identify enriched Gene Ontology (GO) biological process terms as previously described.[31]

2.2.10. Determination of Hepatic Lipogenic Enzyme Activities

Activities of lipogenic enzymes (fatty acid synthase (FASN), EC 2.3.1.85; glucose-6-phosphate dehydrogenase (G6PD), EC 1.1.1.49; malic enzyme (ME), EC 1.1.1.40; 6-phosphogluconate dehydrogenase (6PGD), EC 1.1.1.44) in the livers were determined for rats from all groups ($n = 12$/group). For determination of the enzymatic activities, liver cytosolic fractions were prepared, details of which are provided in Section 7, Supporting Information. FASN activity was measured by the method of Nepokroeff et al.,[33] by monitoring the malonyl-CoA-dependent oxidation of NADPH at 340 nm. G6PD activity was measured using a commercial assay from Sigma-Aldrich (Taufkirchen, Germany; cat. no. MAK015). 6PGD activity was measured spectrophotometrically at 340 nm by monitoring the oxidative decarboxylation of 6-phosphogluconic acid to ribulose-5-phosphate with simultaneous reduction of $NADP^+$ to NADPH.[34] ME activity was determined by incubation of the cytosolic fraction in the presence of malate and $NADP^+$, monitoring the reduction of $NADP^+$ spectrophotometrically, and normalizing to total cellular protein, according to Geer et al.[35]

2.2.11. Statistical Analysis

Statistical analyses were performed using the Minitab statistical software (Release 13.0, Minitab Inc., State College, PA, USA). All data were checked for distribution of normality by Anderson–Darling test. In the case that data were not normally distributed, Johnson transformation was applied. Finally, effects of genotype, diet, and their interaction were analyzed by the General Linear Model (Minitab, Release 13.0; GLM) with Tukey's post-hoc comparisons. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Nutritional Characterization of *Pleurotus sajor-caju*

PSC was successfully grown on ISM in a 150 L stirred-tank vessel after which it was nutritionally characterized and the values of all determinations were calculated on a dry weight basis (Table S2, Supporting Information). After 6 days of cultivation, PSC contained 16% protein. In addition, the amounts of lipids (1.5%), ash (5%), and carbohydrates (78%) were quantitated. The dominating fatty and amino acids were linoleic acid (58.3% of the total fatty acids) and histidine (13.9% of the total amino acids), respectively. The amounts of total glucans, β- and α-glucans in PSC were (29.3 ± 0.22)%, (24.8 ± 0.41)%, and (4.52 ± 0.63)%, respectively. A chitin content of (5.9 ± 0.4)% was determined for PSC cultivated on ISM. Ergothioneine concentration in PSC cultivated on ISM resulted in (844 ± 3.44) mg kg^{-1}.

3.2. Performance of the Rats

The body weight at the beginning of the study was similar within the four groups of rats. As expected, final body weights, daily body weight gain and feed intake were influenced by the genotype ($p < 0.05$, Table S3, Supporting Information). However, addition of PSC to the diet did not influence the performance parameters and neither was any interaction between the genotype and the diet seen (Table S3, Supporting Information).

3.3. Hepatic Lipid Accumulation and Hepatic and Plasma Lipid Concentrations

Oil red O-staining of liver sections revealed a marked lipid accumulation in the liver of the OC group in comparison to the lean groups (LC, LPSC), while liver lipid accumulation in OPSC was greatly reduced in comparison to the OC group (**Figure 1**A). Stained liver sections of the groups, LC and LPSC, exhibited a normal parenchyma structure with no significant lipid accumulation (Figure 1A). In accordance with these histological findings, the liver lipid concentrations were markedly higher in the OC group than in the LC and LPSC group, but 52% and 42%, respectively, lower in OPSC than in the OC group ($p < 0.05$, Figure 1B). Likewise, weights of the livers of rats from the OC group were higher than in the LC and LPSC group, but 24% lower in the OPSC group than in the OC group ($p < 0.05$, Figure 1B). Plasma TG levels did not differ between LC, LPSC, and OC rats; however, in OPSC rats, plasma TG concentrations increased by 104% compared with OC rats ($p < 0.05$, Figure 1C). In OC rats, plasma CHOL increased by 124% compared with LC rats ($p < 0.05$, Figure 1C). Plasma CHOL in LPSC was not affected by PSC in comparison to the LC group; however, plasma CHOL of OPSC rats increased by 30% compared with OC rats ($p < 0.05$, Figure 1C). Plasma NEFA levels in the two obese groups (OC, OPSC) were ≈124% higher in comparison to those of the two lean groups (LC, LPSC) and a significant effect of the genotype was seen ($p < 0.05$, Figure 1C). Inclusion of 5% PSC in the diets of lean (LPSC) or obese (OPSC) rats did not have an effect on the plasma NEFA levels. Significant interactions were found between the genotype and diet for all parameters (except NEFA) considered, indicating that effects of the diet were dependent on the genotype (Figure 1B,C).

3.4. Expression of Hepatic Genes Involved in Lipid Metabolism

In order to unravel the mechanisms underlying the strong hepatic lipid-lowering effect of PSC in the obese rats, hepatic expression of genes involved in lipid metabolism were measured by qPCR. As expected, hepatic mRNA levels of lipogenic genes

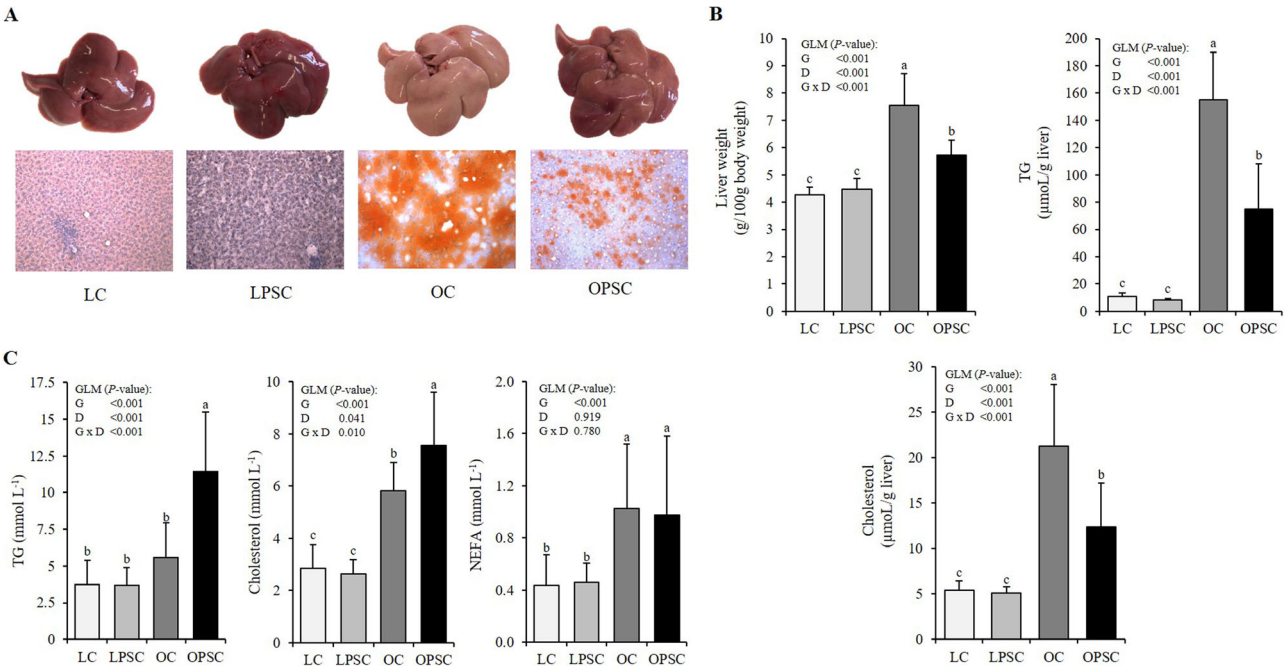

Figure 1. A) Whole livers and Oil red O-stained liver sections; B) liver weights, concentrations of TG, and cholesterol in the liver; and C) concentrations of TG, cholesterol, and NEFA in the plasma of rats fed either the control diet (LC, OC) or the diet supplemented with 5% PSC (LPSC, OPSC) for 4 weeks. A) Images are shown for one animal per group. B,C) Bars represent mean ± SD for $n = 12$ rats/group. Bars without a common letter differ, $p < 0.05$. D, diet; G, genotype; GLM, General Linear Model; G x D, genotype x diet.

(acetyl-CoA carboxylase alpha, *Acaca*; fatty acid synthase, *Fasn*) and cholesterogenic genes (3-hydroxy-3-methylglutaryl-CoA reductase, *Hmgcr*; low density lipoprotein receptor, *Ldlr*; squalene epoxidase, *Sqle*) were lower in the lean groups that in the obese groups ($p < 0.05$, **Figure 2**A) but remained unchanged by the supplementation of PSC. For all the genes considered, there were no interactions between the genotype and the diet, meaning that the effects of diet were independent of the genotype and vice-versa (Figure 2A). Among the genes investigated for lipid catabolism, relative mRNA concentrations of acyl-CoA-oxidase 1 (*Acox1*) was affected by the genotype only ($p < 0.05$, Figure 2B). An interaction of genotype and diet was seen for relative mRNA levels of gamma-butyrobetaine hydroxylase 1 (*Bbox1*), carnitine palmitoyltransferase 1a (*Cpt1a*), and peroxisome proliferator activated receptor alpha (*Ppara*) ($p < 0.05$, Figure 2B) and were highest in group OPSC (except *Ppara*). For *Ppara*, relative mRNA levels were similar in LC, LPSC, and OPSC, while that of OC was significantly lower ($p < 0.05$, Figure 2B). Genes involved in hepatic lipid transport (apolipoprotein B, *Apob*; microsomal triglyceride transfer protein, *Mttp*) were influenced by the genotype ($p < 0.05$, Figure 2C). An interaction effect of the genotype and diet was also seen for *Apob* ($p < 0.05$, Figure 2C), meaning that the addition of PSC decreased the expression of *Apob* in LPSC but tended to increase its expression in the livers of the OPSC.

3.5. Hepatic Transcript Profiling and Bioinformatic Analysis

Since analysis of hepatic genes involved in lipid metabolism did not convincingly explain the strong hepatic lipid-lowering effect of PSC in the obese rats, differential hepatic transcript profiling was performed to get a deeper insight in the mechanisms taking place in the liver of the obese rats that may be responsible for the lipid lowering. As there was no significant effect seen in the hepatic lipid concentrations in LPSC in comparison to LC (Figure 1B), transcript profiling for total RNA samples, only from the obese groups (OC and OPSC, $n = 6$ per group) was carried out. A total of 545 transcripts (164 upregulated, 381 downregulated) were found to be differentially expressed in the liver between OPSC and OC according to the filter criteria applied (FC > 1.3 or < -1.3 and $p < 0.05$). The regulated transcripts are shown as a volcano plot (**Figure 3**) and in a table (Table S5, Supporting Information) with their FCs and *p*-values. The most strongly upregulated transcript was sulfotransferase family 2A member 1 (*Sult2a1*; FC = 4.98), while the most strongly downregulated transcript was cytochrome P450, family 2, subfamily c, polypeptide 55-like (*LOC100361492*; FC = -5.60).

Microarray data of the 16 differentially expressed mRNAs were validated by qPCR. When comparing the FCs of the mRNAs validated between the microarray and qPCR data, the effect direction was the same in all cases, however effect size differed to a mere extent between qPCR and microarray (Table S4, Supporting Information). Statistical analysis of qPCR data showed that all of the 16 validated mRNAs were differentially regulated between OPSC and OC at a significance level of $p < 0.05$ (Table S4, Supporting Information).

To identify biological processes affected by the hepatic genes regulated by PSC, GSEA was performed using GO biological process terms. GSEA of the 164 upregulated genes revealed that the top-enriched GO biological process terms (highest EASE score) were transport, CHOL biosynthetic process and lipoprotein metabolic process (Table S6, Supporting Information). On

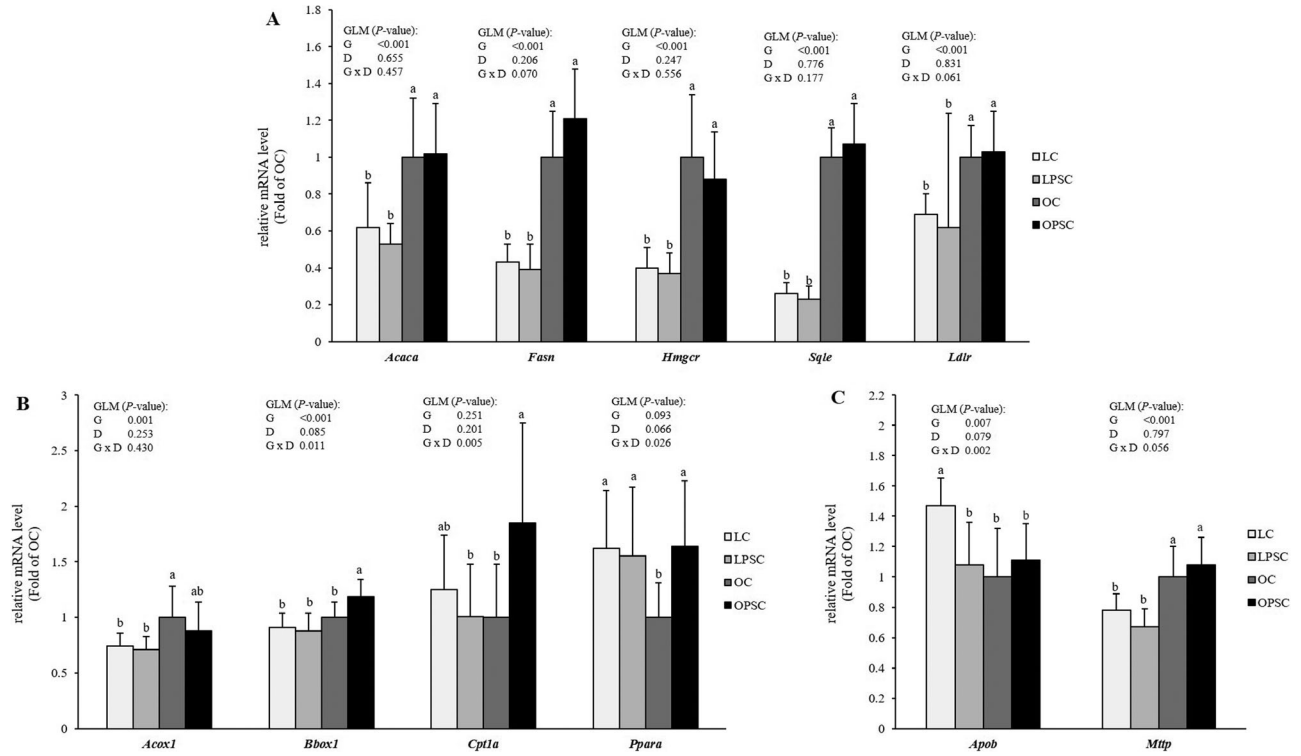

Figure 2. A) Relative mRNA levels of hepatic genes involved in lipid synthesis (*Acaca*, *Fasn*, *Hmgcr*, *Sqle*, *Ldlr*), B) lipid catabolism (*Acox1*, *Bbox1*, *Cpt1a*, *Ppara*), and C) lipid transport (*Apob*, *Mttp*) in rats fed either the control diet (LC, OC) or the diet supplemented with 5% PSC (LPSC, OPSC) for 4 weeks. Relative mRNA levels are expressed as percentage of OC (= 1.0). Bars represent means ± SDs for n = 12 rats/group. Bars without a common letter differ, $p < 0.05$. D, diet; G, genotype; GLM, General Linear Model; G x D, genotype x diet.

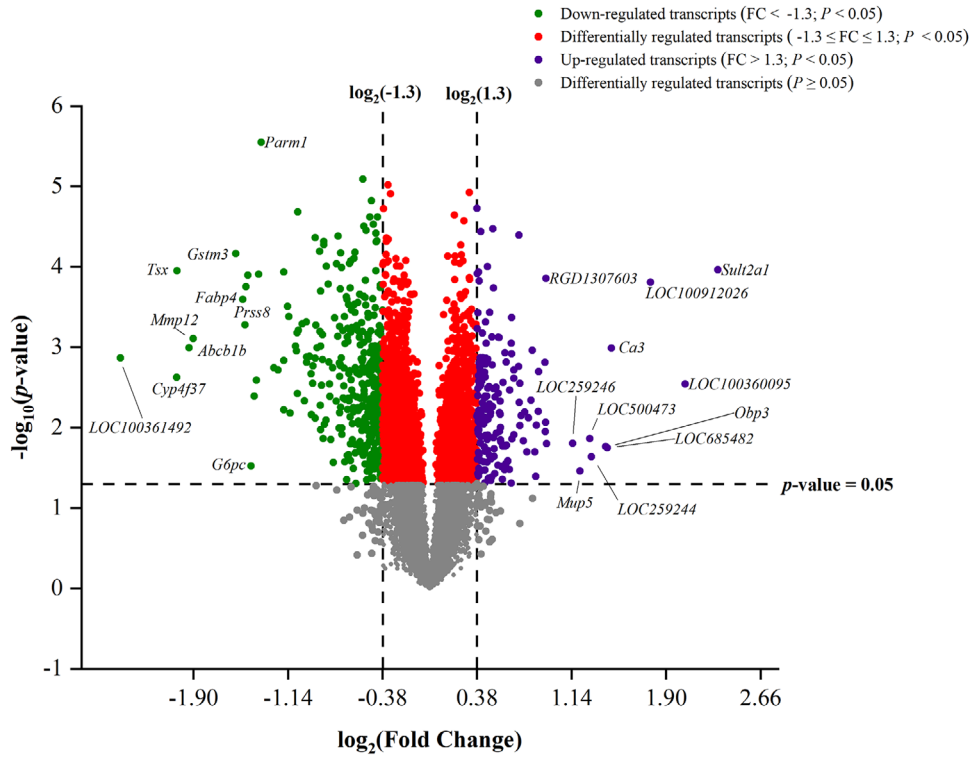

Figure 3. A volcano plot showing all the differentially expressed hepatic transcripts in the OPSC group versus the OC group.

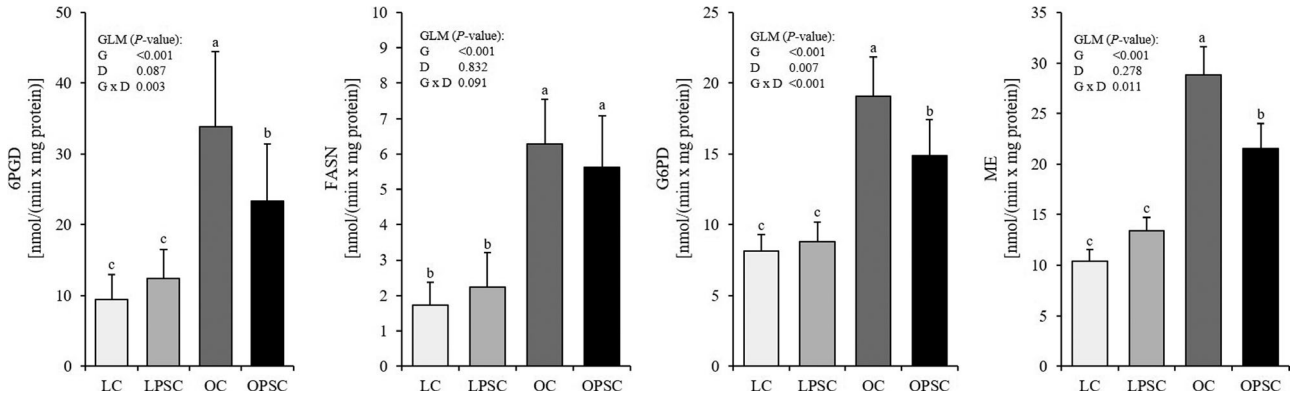

Figure 4. Activities of hepatic lipogenic enzymes of rats fed either the control diet (LC) or the diet supplemented with 5% *Pleurotus sajor-caju* (LPSC) and obese rats fed either the control diet (OC) or the diet supplemented with 5% *Pleurotus sajor-caju* (OPSC) for 4 weeks. Bars represent means ± SDs for $n = 12$ rats/group. Bars without a common letter differ, $p < 0.05$. D, diet; G, genotype; GLM, General Linear Model; G x D, genotype x diet.

the contrary, GSEA of the 381 downregulated genes showed that the top-enriched biological process terms (highest EASE score) were, response to drug, ageing, and wound healing (Table S7, Supporting Information).

3.6. Hepatic Enzyme Activities

Since the activity of proteins may be altered after translation due to the addition or removal of functional groups or by chemical modification, we measured the activities of hepatic enzymes regulating lipogenesis in the cytosolic fraction by spectrophotometry. As expected, the activities of lipogenic enzymes including 6PGD, FASN, ME, and G6PD were significantly higher in the obese Zucker rats (OC, OPSC) than in the rats from the lean groups (LC, LPSC) ($p < 0.05$, **Figure 4**). However, the activities of 6PGD, G6PD, and ME were significantly decreased in the livers of the obese rats fed the 5% PSC supplemented diet ($p < 0.05$, Figure 4) in comparison to the OC group. This decrease was attributed to the interaction of the genotype and diet ($p < 0.05$, Figure 4). Effect of the diet was only observed on G6PD activity ($p < 0.05$, Figure 4). The activity of FASN in the liver was lower in the OPSC group in comparison to the OC group, but this reduction was not significant (Figure 4).

3.7. Expression of Hepatic Genes Involved in Inflammation and Concentrations of Inflammatory Cytokines in the Plasma

Hepatic steatosis is characterized by fat deposition in the liver and is associated with liver inflammation and an increase in proinflammatory markers in the plasma. After seeing the reduction in hepatic lipid concentrations in OPSC and to investigate whether PSC has an influence on liver inflammation and circulating proinflammatory cytokines, we determined the concentrations of TNF-α and IL-6 in the plasma, hepatic mRNA concentrations of two proinflammatory cytokines (tumor necrosis factor, *Tnf*; interleukin 1 beta, *Il1b*), two acute phase proteins (haptoglobin, *Hp*; C-reactive protein, *Crp*), two chemokines (C-C motif chemokine ligand 2, *Ccl2*; C-C motif chemokine ligand 3, *Ccl3*) and intercellular adhesion molecule (*Icam1*). The concentration of TNF-α in the plasma was significantly influenced by the genotype ($p < 0.05$, **Figure 5**A). Rats from the OC group had higher concentrations of TNF-α in the plasma in comparison to the rats from the LC and LPSC group ($p < 0.05$, Figure 5A). As a result of the influence of the interaction between the genotype and the diet, rats belonging to group OPSC had significantly reduced plasma TNF-α concentration in comparison to rats from the OC group ($p < 0.05$, Figure 5A). The concentration of circulating IL-6 remained uninfluenced by genotype, diet or the interaction of these two factors (Figure 5A). Genotype had an influence on the relative mRNA concentration of all the genes (*Il1b*) considered ($p < 0.05$, Figure 5B,C). The relative mRNA concentrations of these genes (*Crp*) were increased in OC when compared to LC and LPSC, but reduced in OPSC compared to OC (Figure 5B,C). For *Crp*, relative mRNA levels were higher in the lean group than in the obese groups ($p < 0.05$, Figure 5B). There was also an influence of the diet on the hepatic relative mRNA expressions of *Ccl2, Ccl3* and *Tnf*. Addition of PSC decreased the expression of these genes, irrespective of the genotype ($p < 0.05$, Figure 5C). An interaction was observed between the genotype and diet for *Hp, Icam1,* and *Tnf* ($p < 0.05$, Figure 5B,C). The decrease in the expression of these genes by including 5% PSC was greater in the obese rats than in the lean rats. There was no effect of the genotype, diet or their interaction on the relative mRNA expression levels of *Il1b* among the 4 groups (Figure 5C).

4. Discussion

Interest in edible mushrooms has consistently grown because of the myriad of health promoting actions they exert. Although beneficial effects of fruiting bodies of *Pleurotus* spp.[36] have been reported, limited information is available pertaining to the health effects of the whole mycelium produced in submerged cultures. In the present study, we demonstrated the bioconversion of ISM, a low value agricultural side stream, to an improved protein source by PSC and its influence on metabolic health of Zucker rats.

The first stage of the study was the biotechnological production of PSC and its chemical analyses, in order to formulate nutrient-adequate diets for laboratory rats. PSC was produced using ISM as the sole carbon source in a 150 L fermenter. This offered the advantages of a short production cycle, reduced space usage

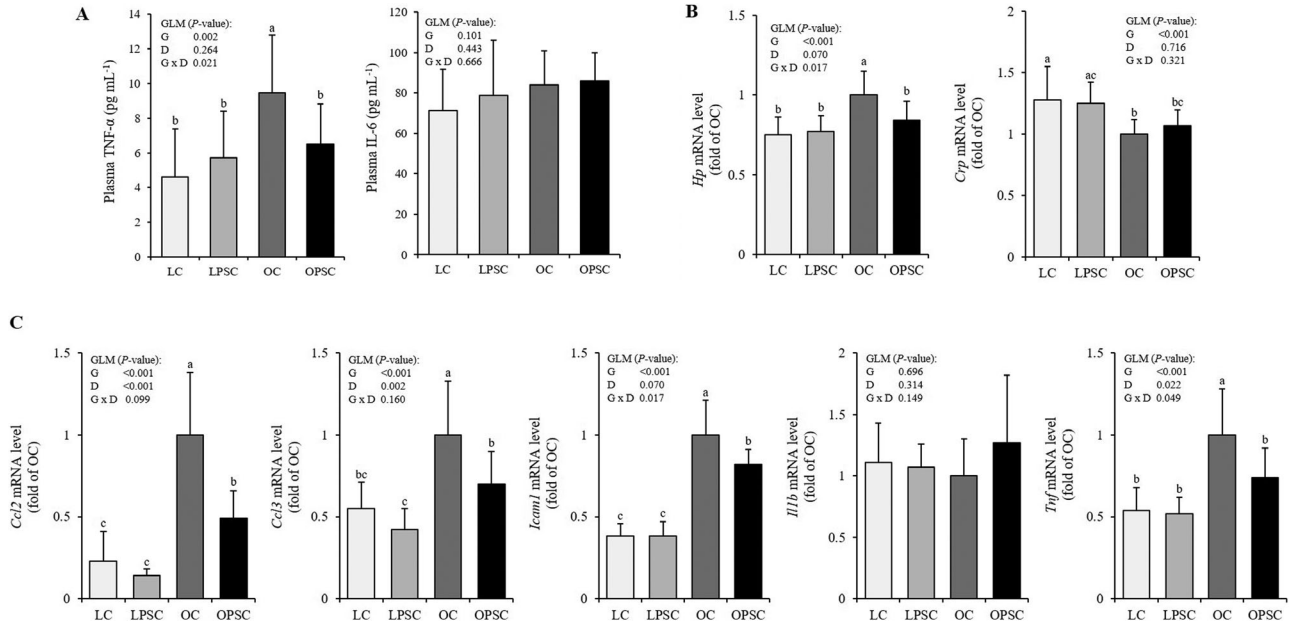

Figure 5. A) Concentrations of proinflammatory cytokines (TNF-α and IL-6) in the plasma, B) relative mRNA levels of acute phase proteins (*Hp*, *Crp*), and C) pro-inflammatory genes (*Ccl2*, *Ccl3*, *Icam1*, *Il1b*, *Tnf*) in the livers of rats fed either the control diets (LC, OC) or the diet supplemented with 5% PSC (LPSC, OPSC) for 4 weeks. Relative mRNA levels are expressed as percentage of OC (= 1.0). Bars represent means ± SDs for n = 12 rats/group. Bars without a common letter differ, $p < 0.05$. D, diet; G, genotype; GLM, General Linear Model; G x D, genotype x diet.

and valorization of an agricultural side stream. Carbohydrates accounted for majority of the macronutrients in PSC (78%) and were close to those reported for *P. ostreatus* grown on various agri-food by-products.[37] The protein content of PSC (15.7%) was also in accordance with ranges mentioned in the literature for other *Pleurotus* species submerged cultivated with different carbon sources.[38] All essential amino acids including cysteine and methionine were quantified in PSC. While previous studies show that the essential amino acid present in highest quantities in *Pleurotus* spp. is either leucine[39] or phenylalanine,[38] the amino acid with the highest concentration for PSC used in this study was histidine. On analyzing PSC's crude ash content (4.8%), it was observed that values were lower than the ash content described in the literature for fruiting bodies of different *Pleurotus* species.[40] The low lipid content (1.5%) of PSC was also in accordance with the lipid content evaluated for submerge cultivated *P. pulmonarious* using different carbon sources.[38] Unsaturated fatty acids remained predominant in PSC. Linoleic acid (58.3%) accounted for most of the fatty acids in PSC which was in good agreement with values reported in literature for most *Pleurotus* strains.[38,41] *Pleurotus* spp. are acknowledged to be one of the most important sources of glucans, making them a potential source of dietary fibre.[42] Glucan contents of PSC were similar to the glucan range reported for other *Pleurotus* species.[43] The PSC chitin content (5.9%) was also similar to that reported for the mycelia of *P. sapidus* grown on apple pomace, but ≈5% lower to that reported by Cheung.[16,44] Mushrooms were recently discovered to be the primary source of ergothioneine which is known for its antioxidant properties.[45,46] Ergothioneine content of PSC was in range with the concentrations reported for other mushrooms, but lower than those reported for fruiting bodies of the *Pleurotus* species.[45] The chemical composition of mushrooms varies, according to the species, growth conditions and composition of the culture medium, which may be the reason for the variability of the chemical composition of PSC seen in this study.[38] Nonetheless, the nutritional composition of PSC grown on ISM indicates it to be a rich source of bioactive compounds.

The intervention period of 4 weeks was chosen because previous studies showed that this time period is sufficient to induce the development of hepatic steatosis and to show inhibitory effects on steatosis development in response to different dietary interventions in obese Zucker rats.[47,29] The rationale for supplementing 5% PSC in the diets of Zucker rats in this study was that in previous studies, inclusion of 5% of different *Pleurotus* fruiting bodies in the diet caused lipid-lowering effects in hyperlipidemic rats.[25,23] The extrapolated HED is approximately tenfold lower than that administered to the obese Zucker rats in the present study considering an average body weight of 449 g per rat and an average feed consumption of 30.6 g per day, which relates to a dose of 3.41 g PSC per kg body weight. Regarding this, a 70 kg person would have to ingest ≈210 g PSC, which is possible in theory if the PSC is taken up via a dietary supplement consisting of pure PSC but is not realistic considering that such a person normally consumes about 500 g food (dry matter) per day. In view of this, the PSC dose tested in our study can be regarded as rather high. Whether or not such as PSC dose can cause adverse effects in humans, cannot be answered and thus future studies are required investigating the effects of a comparable PSC dose in humans. Interestingly, a human study that evaluated the effects of an oyster mushroom (*P. ostreatus*) on blood lipid levels in moderate untreated hyperlipidemic humans is available.[48] In this study, consumption of 30 g dried fruiting bodies of the oyster mushroom in the form of a soup, by 10 subjects with a mean weight of 70 kg, for 21 days, decreased their blood triacylglycerol

concentrations and oxidized low density lipoprotein levels significantly, and showed a significant tendency in lowering total CHOL values.[48] Our extrapolated HED is in line with this study. Therefore, although it is questionable, one might still speculate that PSC exerts its anti-steatotic effects in humans at our extrapolated HED. However, this also remains to be demonstrated in future studies. Nevertheless, the observation that the relatively short intervention period of 4 weeks caused pronounced anti-steatotic effects in the obese rat model is indicative of the high efficacy of this biotechnologically produced PSC mycelial biomass.

In accordance with the hypothesis underlying this study, we observed that supplementation of diets with 5% PSC altered metabolic health in obese Zucker rats (OPSC) by lowering the accumulation of hepatic lipids. This implicates that PSC exerts anti-steatotic effects in the livers of the obese Zucker rats (OPSC). However, these effects were not observed in the lean rats fed PSC (LPSC). Histological observations from Oil red O staining of the rat livers were in agreement with the reduced liver lipid concentrations. Contrary to the effects observed in the liver, PSC feeding increased the plasma lipid levels of the rats. Hypolipidemic effects of oyster mushrooms in rats have been reported before.[23,49] Hypercholesterolemic female Sprague–Dawley albino rats administered 5% powdered *P. eryngii* fruiting bodies, but for a longer period of time caused a lowering of plasma lipids after 6 weeks.[22] Similarly, supplementation of 5% powdered *P. ostreatus* fruiting bodies in the feed of diet-inducedhypercholesterolemic Wistar rats lowered plasma CHOL after 7 weeks[24] and 10 weeks.[25] Either the difference in the animal model used or the shorter intervention period could be the reason for the lack of significant hypolipidemic activity in our study. However, studies in which plasma CHOL in mice increased after *P. ostreatus* feeding are also available,[50] although a potential mechanism that may be responsible for this effect was not specified.

To characterize the hepatic lipid lowering mechanism involved, we focused on the expression of lipid metabolism-related hepatic genes. Of the genes analysed, PSC feeding increased the expression of *Ppara*, *Cpt1a*, and *Bbox1* in obese rats. *Ppara* is the critical transcriptional regulator of lipid catabolism, an increase of which reflects enhanced fatty acid oxidation.[51] *Cpt1a* is a key enzyme for the regulation of long-chain fatty acid β-oxidation in the liver mitochondria.[52] Further, the last reaction of the L-carnitine synthesis pathway, which is a key element in fatty acid metabolism, is encoded by *Bbox1*.[53] These findings suggested that an increased hepatic fatty acid oxidation has led to the decrease in liver TG content. Increases in hepatic *Ppara* and *Cpt1a* mRNA levels in mice after feeding of basidiomycetes have also been documented earlier.[50,54] qPCR analysis of hepatic mRNA levels of genes involved in lipid synthesis and lipid export did not reveal differences between obese rats fed diets without or with PSC. This suggested that the decreased hepatic lipid concentrations and the increased plasma lipid concentrations were not caused by a decreased hepatic lipid synthesis and/or an increased hepatic lipid secretion.

Since qPCR analysis of the abovementioned hepatic genes involved in lipid metabolism could not convincingly explain the lipid-modulating effect of PSC, genome-wide transcript profiling was further applied. A large number of genes were identified as differentially regulated by addition of PSC, which may partially be attributed to the mild filter settings applied. One striking finding of the hepatic transcriptome analysis was that among the top 10 upregulated genes by PSC, there were 8 genes that belonged to the family of major urinary proteins (MUPs) or α_{2U}-globulins. The MUPs, also referred to as α_{2U}-globulins, are hepatic proteins excreted into the urine of adult male mice and rats.[55] MUPs are detected by the main and accessory olfactory systems and trigger adaptive behavioral and developmental processes.[56] Recently, they have also been mentioned as metabolic signals that regulate glucose and lipid metabolism.[56,57] Hepatic lipid metabolism involves several pathways that are at least in part, inter-dependent, and "cross-regulated."[58] It is possible that the upregulated hepatic MUPs in OPSC rats also regulated hepatic lipid metabolism in this study in a yet unclear way and caused a strong reduction of hepatic lipid concentrations.

To further interpret the biological meaning of the differentially regulated genes in the hepatic transcriptome of obese rats, GSEA was performed. GSEA for the upregulated genes showed an involvement of pathways in triglyceride biosynthesis and CHOL transport. The enrichment of these biological processes is a highly interesting observation which is attributable to transcripts involved in de novo lipogenesis, including diacylglycerol O-acyltransferase 2 (*Dgat2*), sterol regulatory element binding transcription factor 1 (*Srebf1*), and *G6pd*. In contrast to our results from qPCR analysis, these results suggested that hepatic lipid synthesis is increased by PSC supplementation. Hepatic transcripts encoding apolipoprotein A4 (*Apoa4*) and apolipoprotein A1 (*Apoa1*) were also upregulated in the OPSC group. *Apoa1* is the main structural and functional component of HDL associated with atherosclerosis protection.[59,60] *Apoa1* modulates HDL remodeling through the actions of a cholesteryl ester transfer protein, and may inhibit CHOL transfer between HDL and other lipoproteins, leading to an increase in HDL composition.[60] In our study, hepatic expression of *Apoa1* was increased by PSC which may have helped to maintain the structural and functional protein component of HDL and contributed to the increase in total plasma Chol levels in OPSC. The *Apoa4* gene is expressed in intestine and liver. It is associated with active intestinal lipid absorption and chylomicron assembly[61] and is linked tightly with hepatic triglyceride export due to its ability to promote VLDL particle expansion.[62] The increased *Apoa4* expression in OPSC may have caused an increased export of hepatic triglycerides and CHOL into the blood stream, explaining the significantly increased plasma lipid levels of OPSC compared to OC. This impact of PSC on the hepatic lipogenic and lipolytic pathways suggests that its addition to the diets of obese Zucker rats increases the total amount of hepatic triglycerides but decreases their accumulation by secretion and oxidation. Since a net retention of hepatic lipids, mostly as triglycerides, is a prerequisite for the development of hepatic steatosis,[63] the hepatic lipid lowering effect of PSC may be explained in a lipid synthesis—mobilization and oxidation mechanism more than on accumulation. However, liver free fatty acid uptake is also increased in metabolic syndrome, in a way that affects their accumulation in the liver.[64] The fatty acid binding protein 4 transcript, a protein upregulated in metabolic syndrome, was identified as one amongst the ten most downregulated genes in the OPSC group. This FABP isoform is expressed in adipose tissue, but has been also associated with hepatic fat accumulation.[65] The overrepresented GO terms associated with the downregulated genes in the liver demonstrate that PSC af-

fects metabolism in an unspecific manner via altering gene expression in a variety of pathways.

To clarify if the reduced liver lipid concentrations are due to the inhibition of hepatic lipid synthesis, we measured the activities of a few hepatic lipogenic enzymes. NADPH is an important cofactor required for FA synthesis, and is provided in a reaction catalyzed either by ME or via the pentose phosphate pathway. ME and two enzymes of the pentose phosphate pathway, 6PGD and G6PD, showed decreased activities in the livers of the OPSC rats in comparison to the OC rats. *G6pd* was also detected in the list of the upregulated transcripts identified by the transcript profiling. Since an impairment of enzyme activities can cause a compensatory up-regulation of genes encoding these enzymes, it is possible that the opposing effects of PSC supplementation on *G6pd* mRNA and G6PD activity are explained by such a feedback regulatory mechanism. However, the mechanism how G6PD function in the liver is impaired by PSC remains open for further investigations. The same could apply to *Dgat2*, which was also found to be upregulated in the livers of PSC fed obese rats. Thus, it also remains to be shown if the hepatic activity of DGAT2 was also reduced in the OPSC rats. Of note, previous research has reported reduced hepatic enzyme activities of G6PD and ME in diet-induced obese rats in response to the addition of *Sparassis* mushrooms to their diets which was partially responsible for the decrease in hepatic lipid levels of these rats.[66] Further, hepatic enzyme activity of ME was also reported to be significantly reduced in Mukitake-fed *db/db* mice.[67] Alleviation of hepatosteatosis in obese Zucker rats by PSC may partially be attributed to the suppressed fatty acid synthesis in these rats.

A recently proposed theory for steatohepatitis is the "two-hit" hypothesis which postulates that the first "hit" is hepatic triglyceride accumulation and these lipid-laden hepatocytes then become more susceptible to a second "hit," that is, injury by inflammatory cytokines, such as TNF-α and CCL2.[67,68] Hepatic synthesis of acute phase proteins is greatly induced in metabolic syndrome disorders triggered by pro-inflammatory cytokines.[69] The observed reduction of plasma levels of TNF-α and the downregulation of mRNA levels of inflammatory cytokines, *Hp* and *Icam1*, in the liver of rats fed PSC are indicators of inhibition of hepatic inflammation. On the other hand, there was no effect on plasma IL-6 levels, even between the lean and obese control groups.

Mushroom enriched diets have previously been reported to show hepatic lipid metabolism-modulating properties. For examples, Mukitake mushroom was found to have suppressive effects on hepatic lipogenesis, and *Pleurotus eryngii* dietary fiber was also reported to have suppressive effects on high-fat-induced steatotic liver formation in rat.[70] Several studies have also suggested that mushrooms exhibit anti-inflammatory activity by modulating the production of inflammatory cytokines.[71] Ergothioneine has been shown to inhibit TNFα-mediated activation of NF-κB in epithelial cells and may be a potential therapy to inhibit the chronic inflammatory response.[72] These multitudinous benefits may also be attributed to the abundance of other bioactive molecules that mushrooms harbor, like phenolic compounds, ergosterol, and tocopherols, among others.[73] Various animal studies have also demonstrated that consumption of mushrooms (fruiting bodies) significantly reduces obesity.[74,75] It would also be interesting to study whether the biotechnologically produced PSC used in this study may have similar, lipid-lowering effects in the adipose tissue as seen in the liver of the OPSC group. The size of the adipose tissue fat stores positively correlates with plasma NEFA levels,[76] which largely explains the increased plasma NEFA levels in the obese rats compared to the lean rats. PSC supplementation did not affect plasma NEFA levels. Hence, we concluded that PSC had no effect on the adipose tissue size of the obese Zucker rats in this study. A lack of effect of PSC supplementation on adipose tissue size can also be assumed from the observation that body weights did not differ between the two obese groups. Nevertheless, we cannot exclude that PSC caused anti-inflammatory effects in the adipose tissue like in the liver, which requires further studies. There is a previous animal study which reports suppressed visceral fat accumulation in rats fed a high-fat diet supplemented with a mushroom mixture, despite insignificant changes in body weights or weight gain, in comparison to control rats fed only the high-fat diet.[74] This suppressed fat accumulation was due to inhibition of fatty acid synthesis, promotion of lipolysis of visceral fat, suppressed adipocyte enlargement, and proliferation of lactic acid and short chain fatty acid producing bacteria which promote energy metabolism in the rats.[74] Also, in view of the strong link between gut microbiota, obesity, metabolic health,[77] and the high glucan content in PSC, it is very likely that at least some of the metabolic effects of PSC may be due to the regulation of gut microbiota. Studying the changes in gut microbial population by sequencing the 16S rRNA genes and quantitation of microbial fermentation products (short chain fatty acids, bile acids) may provide further insight into the role of PSC dietary fibers in modulation of the gut microbiota and alleviation of hepatic steatosis.

To conclude, the data presented in this study support our primary hypothesis that upcycling of an agricultural side stream to a biotechnologically produced alternative protein by PSC alters metabolic pathways in the liver. Moreover, it has a positive influence in decreasing liver inflammation and lipid accumulation. Although we described differences in hepatic gene expression profile, the pathways that are responsible for increasing plasma lipids are still uncertain. Despite unaltered expression of their genes, enhanced circulating lipids could be a result of increased levels of proteins responsible for lipid secretion. Since there has been a long-standing view regarding the positive association of hyperlipidemia with a risk of atherosclerotic cardiovascular diseases, care must be taken in interpreting these results. Further long-term feeding studies including the use of different animal models must be carried out to evaluate the effects of PSC on circulating lipid concentrations. While dietary fibers are the most likely candidate for these effects, other constituents, such as antioxidants (polyphenols and ergothioneine), and proteins may play an important role. Nonetheless, despite the limitations of this study, the marked metabolic effects seen in our animal model as a result of PSC supplementation, suggest its potential nutritional role in exerting anti-steatotic and anti-inflammatory effects in the hepatic tissue.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Data Availability Statement

The hepatic GeneChip microarray profiling data that support the findings of this study are openly available in NCBI's Gene Expression Omnibus public repository with GEO accession no. GSE151882. The remaining data that back the results of this study are available from the corresponding author upon reasonable request.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

K.E. and H.Z. designed research; G.M., D.K.G., S.M., J.A., and G.W. performed experiments and analyzed the data; G.M., R.R., H.Z., and K.E. wrote the paper. All authors read and approved the final manuscript.

Keywords

alternative protein, basidiomycetes, biotransformation, hepatoprotective, transcriptomics

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