Effects of supplementation on the mycelial ergosterol content of *Agaricus bisporus* grown on media formulated with olive oil subproducts.

Gonzalo Falcón-García[1], Pilar Carbonero-Aguilar[2], Olga Cremades[3], Juan Bautista[4]*

[1]Department of Biochemistry and Molecular Biology, Faculty of Pharmacy. Universidad de Sevilla, 41012-Sevilla, (Spain)
[3]Escuela Universitaria de Osuna. Osuna, 41640-Sevilla, (Spain)

***Abstract*—** Supplementation is one of the most relevant procedures for mushroom growth modulation. Little is known about the influence of supplementation on the concentrations of metabolites with potential health benefits. Experiments on compost require long cultivation times. Similar composition has been detected in fruiting bodies and mycelia. Therefore, the mycelial composition can be assumed to be similar to that of the fruiting bodies. This study examines the effect of supplementing a minimal defined growth medium with components derived from olive oil industry subproducts on *A. bisporus* mycelial composition, primarily ergosterol, when grown on minimal defined liquid and solid media in an attempt to obtain a higher concentration of ergosterol (pro-vitamin D2).

A. bisporus supplemented with alperujo meal (ALPM) and olive leaf meal (OLM) led to higher ergosterol content than that of the fungi grown in non-supplemented media (5.64±0.47, 6.60±0.86 and 4.08±0.53 mg/g p.s. in MDLm and 5.36±0.39, 6.79±0.41 and 4.22±0.43 mg/g p.s. in MDSm). Western blotting was used to validate the cultivation results. Three proteins (ERG2, ERG6, and EGR11) involved in the ergosterol biosynthetic pathway were significantly upregulated, indicating the importance of supplementation to ergosterol biosynthesis.

This report represents the first comprehensive study on the protein expression profiling of supplementation studies directed to improve metabolites with potential health benefits in *A. bisporus*. It provides new insights and a better understanding of the development of cultivation processes directed to increase ergosterol biosynthesis. These results could be used to obtain mycelia with higher vitamin D2 content after irradiation with UVB light.

***Keywords*— *Agaricus bisporus*, cultivation medium supplementation, ergosterol, olive leaf meal, two-phase pomace.**

I. INTRODUCTION

Until the last decades of the XX century, the growth of mushrooms on an industrial scale was based more on an art of cultivation than on scientific knowledge. It is known that mushroom growth is influenced by the composition of the growth media [1]. Supplementation is one of the most relevant of the different procedures used to modulate mushroom growth. This practice consists of adding specific substances to compost, during composting to increase nutrient availability and/or active substances, which, when consumed, improve performance and/or quality and probably increase the production of metabolites with health benefits. This technique was introduced in the 1960s [2]; [3]; [4], and from a practical point of view, some important aspects should be considered prior to its application, such as the types of nutrients required, the most suitable application time and economic costs and profits [5]. Most of the relatively few studies published on this topic focused on the productivity of the process. These processes result in yields that generally increase by 10–20% and occasionally by more [6], but little is known about the influence of supplementation on the concentration of metabolites with health benefits, such as ergosterol, ergothioneine, glucans, and chitin. Among the different raw materials that can be used for these purposes are agroindustrial subproducts such as cereal grain brands and meals, oilseed meals, cottonseed meal, and peanut oil and its derivatives, which contain varying amounts of the basic nutritional biomolecules, including carbohydrates, proteins, and lipids. In this study, we examined two agroindustrial subproducts of the olive oil industry that are highly abundant in southern Spain: two-phase pomace or alperujo (ALP) and olive leaf (OL), and its derivatives: alperujo meal (ALPM), olive leaf meal (OLM) and olive leaf hydroalcoholic extract (OLHAE).

Experiments leading to fructification (mushrooms) with edible fungi grown on compost require long cultivation times, and since the same components and metabolites with health benefits have been detected in the fruiting bodies and the mycelia

[1], the mycelial composition can be assumed to be similar to that of the fruiting bodies. Therefore, in this study, we examined the effect of supplementing minimal defined growth medium with different compounds derived from olive oil industry subproducts, ALPM, OLM and OLHAE, on *Agaricus bisporus* (*A. bisporus*) mycelial composition, primarily ergosterol. *A. bisporus* was grown on liquid and solid media as a preliminary step to the study on fruiting bodies after growth on compost supplemented with the same supplement, in an attempt to obtain mushrooms with higher ergosterol (pro-vitamin D2) concentration.

II. MATERIAL AND METHOD

2.1 Mushroom

The mushroom species used in this study was *A. bisporus* (J.E Lange) Imbach strain CBS 57166, obtained from Colección Española de Cultivos Tipo (CECT), Universidad de Valencia (Spain). The mycelia were grown and maintained on potato dextrose agar (PDA) plates at 28ºC. *A. bisporus* was maintained by replication onto new media once a week to keep the mycelia actively growing.

2.2 Agroindustrial subproducts and chemicals

The raw materials or agroindustrial subproducts and the supplements used in this study are shown in **Table-1**. ALP, the solid liquid waste or two-phase pomace generated by the two-phase method of olive oil extraction [7] and OL from Arbequina olive trees were supplied by the Instituto de la Grasa of Seville (Spain). ALPM, OLM and OLHAE were prepared in our laboratory using standard procedures. Briefly, ALP was dried by recirculating air at 50ºC in a drying tunnel until the weight was constant, and dried ALP was milled in a cutting mill (Retsch SM 100, Haan, Germany) and sieved through a 0.25 mm mesh to obtain the ALPM. Similarly, the OL was dried and ground and contained OLM.

2.3 Cultivation media

A. bisporus was grown in two types of cultivation media: minimal defined liquid medium (MDLm) and minimal defined solid medium (MDSm). The composition of MDLm was as follows: 0.5 g/L dextrose, 2.5 g/L potato extract, 0.45 g/L urea, $MgSO_4 \cdot 7\ H_2O$, 590 mg/L, $CaCl_2 \cdot 2\ H_2O$, 602 mg/L and 20 mg/L of mineral solution, pH 5.6. The mineral solution composition was as follows: $FeSO_4 \cdot 7\ H_2O$, 250 mg/L, $MnSO_4 \cdot H_2O$, 80 mg/L, $ZnSO_4 \cdot 7\ H_2O$, 70 mg/L, and $CoCl_2 \cdot 6\ H_2O$ 100 mg/L. The composition of the MDSm was similar but contained 15 g/L of agar. The cultivation media supplemented with ALPM, OLM and OLHAE used in this study is shown in **Table-3** and **Table-4**. Cultivation in MDLm was conducted in 500 mL flasks with 200 mL culture media at 28ºC, pH 5.6 and 120 rpm in a shaker for 7 days. Five millilitre samples were removed every day and analysed for biomass and ergosterol. Cultivation in MDSm was carried out in Petri dishes of 9 cm in diameter containing 20 mL culture media at 28ºC and pH 5.6 in a cultivation oven for 7 days. Samples (one Petri dish) were removed each day and analysed for biomass and ergosterol. The physical and chemical characteristics of the supplements used in this study are shown in **Table-2**.

TABLE 1
RAW MATERIALS AND SUPPLEMENTS USED ON MUSHROOM (*A. BISPORUS*) GROWTH MEDIA FORMULATION.

Agroindustrial subproduct	Supplement
Alperujo (ALP)	Alperujo meal (ALPM)
Olive leaf (OL)	Olive leaf meal (OLM)
Olive leaf (OL)	Olive leaf hydroalcoholic extract(OLHAE)

TABLE 2
PHYSICAL AND CHEMICAL CHARACTERISTICS OF THE SUPPLEMENTS ASSAYED IN THIS STUDY.

	ALP	OLM	OLHAE
Dry matter[&] (%)	88.7±3.8	93.2±1.1	22.3±3.7
Moisture* (%)	11.3±3.8	6.8±1.1	77.7±3.7
Ash* (%, d.w)	11.4±1.2	9.4±3.1	5.2±0.8
Organic matter[&] (%, d.w.)	88.6±1.2	90.6±3.1	94.8±0.8
Total-N* (%, d.w.)	1.5±0.2	1.9±0.3	3.4±0.4
Protein[&] (N_t x 5.5) (%, d.w.)	8.3±0.9	10.5±1.3	18.7±1.8
Carbohydrates* (%, d.w.)	50.2±3.6	46.6±4.2	59.4±3.8
Lignin (%, d.w.)	21.7±3.2	17.2±1.9	-
Fat* (%, d.w.)	6.8±0.8	5.3±0.6	13.8±1.6
Others[&] (%, d.w.)	1.6±	3.8±	2.9±
Polyphenols (mg/g, d.w.)*	12.4±3.7	23.7±3.6	240.1±15.1
C/N*	9.7±2.4	15.3±3.1	4.1±0.7
pH*	5.7±0.3	6.9±0.2	7.1±0.2

**Experimental data are the mean of at least three experiments; [&]calculated data.*

2.4 Proximate composition of supplements

The moisture content was measured using a moisture analyser (Mettler Toledo, Barcelona, Spain). The total nitrogen content was determined using an AOAC 920.87 semi-micro Kjeldahl method [8] with a conversion factor of 5.5 to transform nitrogen into protein. The ash content was determined using the direct ashing method of AOAC [9]. The fat content was measured using an automated Soxhlet extraction apparatus (Soxtec™ 2050 FOSS, Hillerød, Denmark). Fat was extracted from 3 g of sample with 100 mL of petroleum ether at 90 °C for 180 min, and the defatted sample was collected and dried under nitrogen gas.

2.5 Carbohydrate determinations

The total carbohydrate content of the mycelial samples was determined using the phenol-sulfuric acid method [10], adapted to microplate analysis. Briefly, 1 mg samples were mixed with 1 mL of MilliQ water and stirred for 2 min. Twenty-five microlitres of the mixtures were added to a 96-well plate along with 25 µL of 5% phenol solution (w/v) and 125 µL of concentrated H_2SO_4. The plate was sealed and incubated in a water bath at 80 ºC for 30 min. Sample absorbance was determined using a microplate reader (Biorad Model 680) at 595 nm. A standard curve of D-glucose (0.03 to 1.0 mg/mL) was used for quantification.

2.6 Total phenol content

The total phenol concentration was determined using the Folin-Ciocalteu method according to standard procedures [11]. Gallic acid was used as the standard for quantification.

2.7 Soluble protein determination

Total soluble protein concentration was determined using the Bradford method according to standard procedures using bovine serum albumin as the standard [12].

2.8 Ergosterol determination

2.8.1 Sample extraction

The extraction method used is a modification of the direct hexane extraction method proposed by Shao et al. [13]. Briefly, 2.5 mg of mushroom powder sample was vortexed with 6 mL of hexane for 2 min, centrifuged at 4000 rpm for 15 min, and the supernatant was transferred to a 25 ml beaker. The mushroom residue was then extracted twice using 6 mL hexane each time.

The three hexane phases obtained were pooled and dried under a nitrogen stream. The extract was dissolved in 1.5 mL of methanol. This solution was homogenised in an ultrasonic bath and then filtered through 0.22 µm PVDF filters (VWR, Spain) before chromatographic analysis. To carry out the internal standard (I.S.) calibration method, adequate volumes (µL) of 100 mg L^{-1} standard solution of vitamin D3 was added to the mushroom powder before extraction, so that the final concentration of the I.S. was 1 mg L^{-1}.

2.8.2 Liquid chromatography-mass spectrometry analysis

The analysis of ergosterol and ergocalciferol, using cholecalciferol as an internal standard, was performed in an Acquity ultra-performance liquid chromatography (UPLC) system (Waters, Milford, MA, USA) coupled to a Xevo G2S QTOF mass spectrometer (Waters, Micromass, Manchester, UK) with a dual electro spray chemical ionisation (ESCI) in positive mode. The LC separation was performed using a conditioned autosampler at 5ºC and a Synergi Hydro-RP column (100 mm x 3.00 mm id, 2.5 µm particle size), (Phenomenex, CA, USA), thermostated at 35ºC. The mobile phase consisted of aqueous formic acid solution 0.1% (solvent A) and methanol (solvent B). The gradient elution starts at 1% A, maintaining these conditions for 4 min and then, increasing % A from 1% to 10% in 1 min maintaining conditions for 5 min at a flow rate of 0.5 mL min^{-1}. The injection volume was 5 µL. To avoid cross-contamination, pre and post-inject washes were conducted. MS conditions were as follows: nebulisation gas (nitrogen) 700 l/h, cone gas (nitrogen) 29 L h^{-1}, desolvation temperature 500 ºC, source temperature 150 ºC, corona current was set at 5 mA and sample cone voltage at 40 V. MS spectra were collected in a continuum between 50 and 750 Da with a scan time of 1.55. Ergosterol, ergocalciferol and cholecalciferol were identified using retention times of 9.13 min for ergosterol; 4.68 min for ergocalciferol and 5.06 min for cholecalciferol, as well as the accurate mass of each compound (379.3377 for ergosterol; 397.3462 for ergocalciferol and 385.3463 for cholecalciferol).

2.9 Western blot analysis

Total soluble protein was resolved on 12.5% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) as described by Laemmli [14] and electro-transferred to nitrocellulose membranes. Western blot analysis was performed as described by Haid and Suissa [15]. Key enzymes related to ergosterol biosynthesis were detected using rabbit antisera as the primary antibodies directed against ERG2, ERG4, ERG6 and ERG11 (**Figure 1**). The primary antibodies were detected using peroxidase-conjugated goat anti-rabbit IgG as a second antibody and SuperSignal® West Pico Chemiluminescent substrate solution.

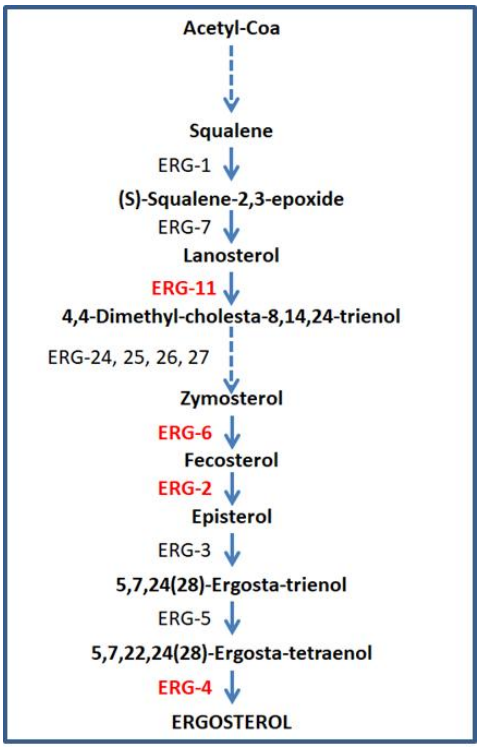

FIGURE 1: REPRESENTATIVE ENZYMES IMPLICATED IN ERGOSTEROL BIOSYNTHESIS IN FUNGI.

(ERG1 − squalene epoxidase; ERG2 − sterol C-8 isomerase; ERG3 − sterol C-5 desaturase; ERG4 − sterol C-24 reductase; ERG5 − sterol C-22 desaturase; ERG6 − sterol C-24 methyltransferase; ERG7 − lanosterol synthase; ERG11 (CYP51) − lanosterol C-14 demethylase; ERG24 − sterol C-14 reductase; ERG25 − sterol C-4 methyl oxidase; ERG26 − sterol C-3 dehydrogenase (C4-decarboxylase); ERG27 − sterol C-3 ketoreductase).

2.10 Statistical analysis

The results are presented as the mean value ± standard deviation (SD). Statistical analyses were performed using a 2-way analysis of variance (ANOVA) with a subsequent Bonferroni post hoc test for pairwise comparisons between various combinations of two groups. A *p*-value of < 0.05 was considered statistically significant. Statistical analyses were performed using the SPSS 16.0 software.

III. RESULTS AND DISCUSSION

3.1 Preparation and characterisation of supplements

The first objective of this study was to collect enough raw materials (agroindustrial subproducts) to obtain supplements for all experiments planned. These included growth on liquid- and solid-minimal medium and, in the future, in compost. Using this strategy reduced the variability in the raw material composition, which can occur when subproducts obtained at different periods of times were used. The agroindustrial subproducts used in this study were ALP and OL, and the supplements derived from them were ALPM, OLM and OLHAE (**Table-1**). These subproducts were selected because they are highly abundant in southern Spain, cheap, and their composition can influence the growth of *A. bisporus* [16]. Although available seasonally, further processing can provide enough supplements to use for a long period of time, such as an entire year. For example, ALPM and OLM can be processed to a stable dry form or into a liquid form or syrup, such as OLHAE, that remains stable for long periods.

The physical and chemical characteristics of the supplements used in this study are shown in **Table-2**. As these results show, the properties and composition of the supplements vary from each other in their stabilised forms, while ALPM and OLM are solids (meals), and OLHAE is liquid. Thus, its composition depends on the components of the raw materials (ALP and OL). Stabilisation is required to keep these supplements from degrading for one year. Therefore, the water content was reduced by air drying and constitutes the primary cost of the process.

ALP is a subproduct from the olive oil industry, generated by oil centrifugation, with a high water content of approximately 60%. After drying, the moisture is reduced to 11.3±3.8%. The dry matter that constitutes 88.7±3.8% of the ALPM is comprised primarily of carbohydrates (50.2±3.6%), lignin (21.7±3.2%), proteins (8.3±0.9%), fat (6.8±0.8%) and ash (11.4±1.2%). OL provides similar results. The fresh leaves contain approximately 15% of water, and after air drying and milling, it can be stabilised as OLM with a moisture content of 6.8±1.1%. The dry matter that constitutes 93.2±1.1% of the OLM is comprised primarily of carbohydrates (46.6±4.2%), lignin (17.2±1.9%), proteins (10.5±1.3%), fat (5.3±0.6%) and ash (9.4±3.1%). OL milling to a fine powder (OLM) allows the hydroalcoholic extraction of bioactive compounds. The extract that occurs after ten-fold concentration by vacuum is a syrup containing a high water content (77.7±3.7%) and, consequently, a lower percentage of dry matter (22.3±3.7%), comprised primarily of carbohydrates (59.4±3.8%), proteins (18.7±1.8%), fat (13.8±1.6%) and ash (8.8±2.3%).

The analysis of the organic matter shows that the level in ALPM (88.6±1.2%) is lower than that in OLM and OLHAE (90.6±3.1% and 94.8±0.8%, respectively) due to its higher ash content.

The fat contents of the three supplements are 6.8±0.8%, 5.3±0.6% and 13.8±1.6% in ALPM, OLM and OLHAE, respectively. The relatively low fat content in ALP (6.8±0.8%) can be explained because developing fats is the primary objective during olive oil production, and minimal fat loss is desired during the oil recovery process. OLM shows the lowest fat content (5.3±0.6%) since the fat content is low in the OL. However, the highest lipid concentration (13.8±1.6%) was found in the OLHAE, since a lipophilic solvent (ethanol) was used in the hydroalcoholic extraction medium.

The protein contents in the ALPM, OLM and OLHAE supplements are 8.3±0.9%, 10.5±1.3% and 18.7±1.8, respectively. These values were obtained by multiplying the respective total nitrogen (N_t) value by the more convenient conversion factor 5.5 for vegetable proteins, rather than by 6.25, which is appropriated for animal proteins [17].

The carbohydrate content was 50.2±3.6%, 46.6±4.2% and 59.4±3.8% in ALPM, OLM and OLHAE, respectively. The primary carbohydrates present in the supplements contain cellulose and hemicellulose with a low concentration of low molecular weight saccharides. The lignin content was 21.7±3.2% and 17.2±1.9% in ALPM and OLM, respectively. OLHAE, due to the nature of its preparation process, does not contain lignin.

The C/N ratio is an important factor for mycelial growth, and fungi grow well when its values are approximately 10 to 12 [18]. Thus, our supplements result in the following values: 9.7±3.4%, 15.3±3.1% and 4.1±1.3% for ALPM, OLM and

OLHAE, respectively. Supplementation with OLHAE could explain the faster growth and higher productivity that *A. bisporus* shows in media supplemented with OLM and ALPM (data not shown). However, the objective of the supplementation in this study was not nutritionally related but instead examined the utilisation of stress-inducing factors that could potentiate the increase in metabolites such as ergosterol and ergothioneine. Thus, substances such as polyphenols and other non-identified metabolites present in the supplements could be of special interest for those purposes. The concentration of polyphenols in the supplements varies from 12.4±3.7 mg/g, 23.7±3.6 mg/g and 240.1±15.1 mg/g in ALPM, OLM and OLHAE, respectively. The higher value observed in OLHAE is related to the extraction and concentration processes that facilitate the extraction of such types of compounds. As we will demonstrate, a relatively high polyphenol content, as shown in ALPM and OLM, induces ergosterol biosynthesis; however, very high content, as is the case with OLHAE, could be less effective, probably due to its action as pro-oxidants rather than as antioxidants [19].

3.2 Effects of supplementation on *A. bisporus* growth and mycelial composition

The modification of MDLm and MDSm was conducted once the supplements had been prepared and characterised (see **Tables-3** and **-4**). Although the primary objective of this study was to examine the effect on the biosynthesis of some components with health benefits, such as ergosterol, the precursor of vitamin D2, and ergothioneine, the effects on biomass production were also considered.

Methods for increasing the productivity of any one metabolite of interest, such as ergosterol, are generally based on two approaches: i) engineering metabolic pathways [20], and ii) utilising a physiological approach to exploit knowledge about the physiology of the mushroom strain and its response to changing environmental conditions [21]. Since the genetics and biochemistry of *A. bisporus* have not been fully developed, we used the physiological approach to study the effect of a group of supplements derived from the olive oil industry (ALPM, OLM and OLHAE) on the synthesis of ergosterol, in an attempt to obtain *A. bisporus* with higher ergosterol concentration.

A. bisporus mycelia were growth on MDLm supplemented with different amounts of ALPM, OLM and OLHAE as shown in **Table-3** at pH 5.5, 28ºC and gentle agitation (120 rpm) for 7 days. As observed from these results, supplementation with ALPM, OLM and OLHAE at 0.1%, 0.5%, 1% and 2% significantly increased the biomass and ergosterol content compared to the non-supplemented control culture. Although the increase in the biomass was more significant in ALPM and OLM, probably due to their higher C/N rates of 9.7±2.4 and 15.3±3.1, with an increased amount of supplementation, the ergosterol concentration reaches its maximum at 1% supplementation. A higher concentration does not lead to the production of more ergosterol, and it reached a plateau at 1% supplementation. Therefore, we selected 1% supplementation for further studies.

TABLE 3
***A. BISPORUS* GROWTH ON MDLM SUPPLEMENTED WITH DIFFERENT SUBSTRATES AND CONCENTRATIONS.**

Culture media	Biomass (mg/L)	Ergosterol (mg/g, d.w.)	n
MDLm (control)	7.8±0.6	4.08±0.53	4
MDLm +ALPM (0.1%)	9.7±0.7	4.86±0.93	4
MDLm + ALPM (0.5%)	11.9±1.1	5.61±0.89	6
MDLm +ALPM (1.0%)	12.2±0.9	5.64±0.47	6
MDLm +ALPM (2.0%)	12.7±1.4	5.59±0.35	4
MDLm + OLM (0.1%)	10.2±0,8	5.14±0.53	4
MDLm + OLM (0.5%)	12.1±1.3	6.23±0.47	6
MDLm + OLM (1.0%)	12.6±0.5	6.60±0.86	6
MDLm + OLM (2.0%)	12.9±2.1	6.62±0.56	4
MDLm + OLHAE (0.1%)	8.8±0,9	4.92±1.33	4
MDLm + OLHAE (0.5%)	9.3±1.2	5.31±0.61	4
MDLm + OLHAE (1.0%)	9.6±1.1	5.87±0.91	4
MDLm + OLHAE (2.0%)	9.9±2.2	5.85±0.78	4

A. bisporus mycelia were grown on MDSm supplemented with different amounts of ALPM, OLM and OLHAE, as shown in **Table-4**, at pH 5.5, 28ºC without agitation and in darkness for 7 days. The results of this experiment were similar. These results show that supplementation with ALPM, OLM and OLHAE at 0.1%, 0.5%, 1% and 2% significantly increase the biomass and ergosterol content in comparison with the non-supplemented control culture. In this case, the biomass also increases more significantly in ALPM and OLM than in OLHAE, probably due to its higher C/N rate, 9.7±2.4 and 15.3±3.1, respectively, which increased with the degree of supplementation. Ergosterol concentration reaches its maximum at 1% supplementation, and a higher concentration of supplements did not result in higher ergosterol content, which reached a plateau at 1% supplementation. Therefore, in this case, we also selected 1% supplementation for further studies.

The expression of several enzymes implicated in ergosterol biosynthesis was studied to investigate the reason for the observed increase in ergosterol biosynthesis and/or accumulation in *A. bisporus* grown in media supplemented with OLM and ALP. For this purpose, we prepared antibodies against four of the key enzymes (**Figure 1**) implicated in ergosterol biosynthesis, ERG2, ERG4, ERG6 and ERG11, to use for a Western blot analysis. Due to the difficulty of purifying these enzymes implicated in the biosynthesis of this phytosterol, we choose a procedure to produce the antibodies based on antibody preparations against synthetic peptides with high immunogenicity [22] towards the different enzymes.

TABLE 4
***A. BISPORUS* GROWTH ON MDSM SUPPLEMENTED WITH DIFFERENT SUBSTRATES AND CONCENTRATIONS.**

Culture media	Biomass (mg/L)	Ergosterol (mg/g, d.w.)	n
MDSm (control)	6.6±0.4	4.22±0.43	3
MDSm +ALPM (0.1%)	8.7±0.8	5.07±0.71	4
MDSm + ALPM (0.5%)	8.9±0.6	5.21±0.33	4
MDSm +ALPM (1.0%)	9.2±0.2	5.36±0.39	4
MDSm +ALPM (2.0%)	9.5±0.5	5.42±0.41	3
MDSm + OLM (0.1%)	8.6±0.6	5.22±0.34	4
MDSm + OLM (0.5%)	8.9±0.5	6.45±0.55	4
MDSm + OLM (1.0%)	9.4±0.7	6.79±0.42	4
MDSm + OLM (2.0%)	9.7±0.3	6.83±0.65	3
MDSm + OLHAE (0.1%)	7.1±0.2	4.53±0.81	4
MDSm + OLHAE (0.5%)	7.3±0.3	4.72±0.65	4
MDSm + OLHAE (1.0%)	7.6±0.5	4.93±1.01	4
MDSm + OLHAE (2.0%)	7.8±0.4	5.13±0.32	3

Our results show that all of the antibodies produced recognised the synthetic peptide by a dot analysis, even at a level of 5 ng (**Figure 2**). However, when we assayed the antibodies against the *A. bisporus* enzymes, only three of them recognised any protein using Western blot analysis, anti-ERG4, anti-ERG6 and anti-ERG11, (Figure-3), probably due to the localisation of the immunogenic peptides at the molecular surface. In contrast, the selected anti-ERG4 peptide could be occulted in the interior of the molecule and thus not recognised by anti-ERG-2.

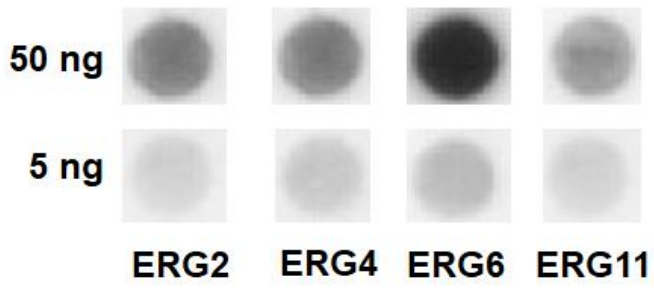

FIGURE 2: DOT ANALYSIS USING 5 AND 50 NG OF SYNTHETIC PEPTIDES AND DETECTION BY THEIR ANTIBODIES (ANTI-ERG2, ANTI-ERG4, ANTI-ERG6 AND ANTI-ERG11).

Western blot analysis also showed that ERG6 (C-24 sterol methyltransferase) and ERG11 (cytochrome P450 lanosterol C-14α-demethylase) were expressed at a higher level than in the control (2.75-fold and 3.52-fold, respectively) in all three supplemented culture media assayed, both in MDLm (**Figure-3**) and MDSm (data not shown) than ERG2 (sterol C-8 isomerase). Although the ERG2 expression level was 1.85-fold higher than that in the control, it is lower than that observed for the other two enzymes analysed. From these results, we concluded that at least three proteins (ERG2, ERG6 and ERG11) involved in the ergosterol biosynthesis pathway were significantly upregulated, indicating the importance of activating ergosterol biosynthesis by supplementation with ALPM, OLM and OLHAE.

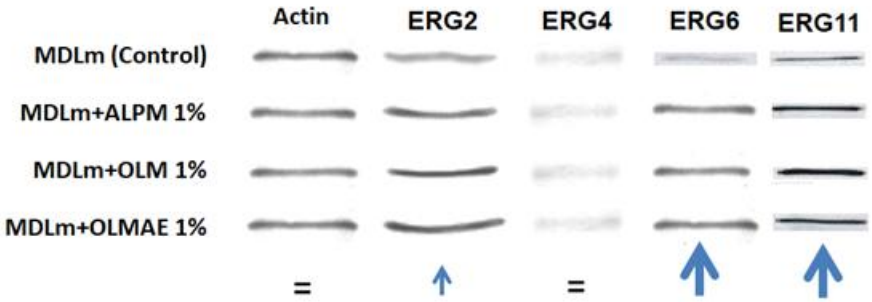

FIGURE 3: WESTERN BLOT ANALYSIS OF *A. BISPORUS* KEY ENZYMES (ERG2, ERG4, ERG6 AND ERG11) IMPLICATED IN ERGOSTEROL BIOSYNTHESIS

IV. CONCLUSION

From these results, we can conclude that supplementation with ALPM, OLM and OLHAE upregulate some of the key enzymes implicated in ergosterol biosynthesis (ERG2, ERG6 and ERG11), but we do not know what compound is responsible for this upregulation and the subsequent increase in ergosterol production. Therefore, more research is necessary to increase our knowledge in this field. To the best of our knowledge, this report represents the first comprehensive study on the protein expression profiling of supplementation studies directed to improve the production of metabolites with potential health benefits. It provides new insights into a better understanding of the development of cultivation processes directed to increase ergosterol biosynthesis, which is a prerequisite for obtaining mushrooms with high vitamin D2 levels, sufficient for a daily dose (15 µg) in one portion (100 g w/w.).

ACKNOWLEDGEMENTS

We are grateful to the Spanish Ministry of Science and Innovation for the financial support of this work (project RTC-2015-4039-2), which has partial financial support from the FEDER funds of the European Union. The authors are grateful to the Centro de Innovación, Tecnología e Innovación de la Universidad de Sevilla (CITIUS, Servicio de Biología and Servicio de Microanalisis) for providing instrumental and technical support. The authors thank Paula Bautista for assistance in the preparation of the manuscript.

REFERENCES

[1] Y. Chen, B. Chefetz, R. Rosario, J. D. H. van Heemst, C. P. Romaine, and P. G. Hatcher, "Chemical nature and composition of compost during mushroom growth". Compost Sciene & Utilization, 2000, 8(4): 347-359

[2] G. Lemke, "Champignon kultur auf nicht kompostiertem Stroh substrat mit Startddungung''. Die Deutsche Gartenbauwirtschaft, 1963, 11: 167–169.

[3] J. W. Sinden, and L. C. Schisler, "Nutrient supplementation of mushroom compost at spawning". Mushroom Sci, 1962a 5: 150–164.

[4] J. W. Sinden, and L. C. Schisler, "Nutrient supplementation of mushroom compost at casing". Mushroom Sci, 1962b, 5: 267–280.

[5] P.E. Randle, "Supplementation of mushroom composts – A review. Mushroom J, 1985, 151: 241–249.

[6] A. Pardo-Giménez, D.C. Zied, M. Álvarez-Ortí, M. Rubio, and J.E. Pardo, "Effect of supplementing compost with grapeseed meal on *Agaricus bisporus* production". J Sci Food Agric, 2012, 92: 1665–1671.

[7] J.A. Alburquerque, J. Gonzálvez, D. García, and J. Cegarra, "Agrochemical characterisation of "alperujo", a solid by-product of the two-phase centrifugation method for olive oil extraction". Bioresource Technology, 2004, 91: 195–200.

[8] AOAC, 1995. Method 920.87. Protein (total) in flour, final action. Official Methods of Analysis of AOAC International. AOAC International, Maryland, USA, p. 32.1.22.

[9] AOAC, 2000. Method 923.03. Ash of flour. Official Methods of Analysis of AOAC International. AOAC International, Maryland, USA, p. 32.1.05.

[10] M. DuBois, K .A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith, "Colorimetric Method for Determination of Sugars and Related Substances". Anal Chem, 1956, 28: 350–356.

[11] R. Prior, X. Wu, and K. Schaich, "Standarised Methods for the Determination of Antioxidant Capacity and Phenolics in Foods and Dietary Supplements". J Agric Food Chem, 2005, 53: 4290–4302.

[12] M. A. Bradford, "Rapid and Sensitive Method for the Quantification of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding". Analytical Biochemistry, 1976, 72: 248–254.

[13] S. Shao, M. Hernandez, J. K. G. Kramer, D. L. Rinker, and R. Tsag, "Ergosterol profiles, fatty acid composition and antioxidant activities of button mushrooms as affected by tissue part and developmental stage". J Agric Food Chem, 2010, 58: 11616-11625.

[14] U. K. Laemmli, "Cleavage of structural proteins during the assembly of the head of bacteriophage T4". Nature, 1970, 227: 680–685.

[15] A. Haid, M. Suissa, "Immunochemical identification of membrane proteins after sodium dodecyl sulfate-polyacrylamide gel electrophoresis". Methods Enzymol, 1983, 96: 192–205.

[16] R. Altier, A. Esposito, F. Parati, A. Lobianco, and M. Pepi, "Performance of olive mill solid waste as a constituent of the substrate in commercial cultivation of *Agaricus bisporus*". Int Biodeter Biodegradation, 2009, 63: 993–997.

[17] F. Mariotti, D. Tomé, and P. P. Mirand, "Converting Nitrogen into Protein—Beyond 6.25 and Jones' Factors", Critical Reviews in Food Science and Nutrition, 2008, 48: 177-184.

[18] A. Pardo-Giménez, D.C. Zied, M. Álvarez-Ortí, M. Rubio, and J. E. Pardo, "Effect of supplementing compost with grapeseed meal on *Agaricus bisporus* production". J Sci Food Agric, 2012, 92: 1665–1671.

[19] L. R. Fukumoto, and G. Mazza, "Assessing Antioxidant and Prooxidant Activities of Phenolic Compounds". J Agric Food Chem, 2000, 48: 3597–3604.

[20] E. Nevoigt, "Progress in Metabolic Engineering of *Saccharomyces cerevisiae*". Microbiol Mol Biol Rev**,** 2008, 72: 379–412.

[21] B. Sonnleitner, O. Käppeli, "Growth of *Saccharomyces cerevisiae* is controlled by its limited respiratory capacity: formulation and verification of a hypothesis". Biotechnol Bioeng, 1986, 28: 927–937.

[22] F. Araujo, S. Tan, D. Ruano, H. Schoemaker, J. Benavides, and J. Vitorica, "Molecular and Pharmacological Characterization of Native Cortical γ-Aminobutyric $Acid_A$ Receptors Containing Both α1 and α3 Subunits". J Biol Chem, 1996, 271: 27902–27911.