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Immunomodulatory Potential of Digestion-Derived Protein and Polysaccharide Fractions from *Pleurotus* spp. Cultivated on Agro-Industrial Residues

Eleni Dalaka [1,*], Panagiota Diamantopoulou [2], Ilias Diamantis [2], Grigorios Lytras [1], Despoina-Eirini Bekiari [1], Ioannis Politis [1] and Georgios Theodorou [1,*]

[1] Laboratory of Animal Breeding & Husbandry, Department of Animal Science, Agricultural University of Athens, 75 Iera Odos, 11855 Athens, Greece; greg.lyt99@gmail.com (G.L.); nteniabekiari4@gmail.com (D.-E.B.); ipolitis@aua.gr (I.P.)

[2] Laboratory of Edible Fungi, Institute of Technology of Agricultural Products (ITAP), Hellenic Agricultural Organization-Dimitra, 1, Sofokli Venizelou, 14123 Lykovryssi, Greece; pdiamantopoulou@elgo.gr (P.D.); idiamantis@aua.gr (I.D.)

* Correspondence: elenidalaka@aua.gr (E.D.); gtheod@aua.gr (G.T.)

Abstract

Lignocellulosic agricultural residues are abundant yet underutilized despite their potential for sustainable bioconversion. This study evaluated spent mushroom substrate (SMS) from *Pleurotus ostreatus* cultivation and roots of leafy vegetables (RLV) as alternative substrates for *Pleurotus* production, using wheat straw as a control. Two species, *P. ostreatus* and *P. citrinopileatus*, were cultivated on different SMS/RLV ratios and the immunomodulatory potential of harvested mushrooms was assessed. Specifically, protein (PE-D-P3 < 3 kDa) and carbohydrate (CE-D) fractions obtained after in vitro digestion were applied to LPS-challenged THP-1 cells and immune-related gene expression was analyzed by qPCR. Both species significantly modulated immune responses. The PE-D-P3 showed a more pronounced immunomodulatory effect, significantly downregulating *IL1B*, *IL6* and *TNF*, whereas the CE-D reduced only *TNF* expression. Substrate composition influenced bioactivity: PE-D-P3 from SMS 80-RLV 20% resulted in the greatest reduction in *IL1B*, *IL6* and *TNF*, while CE-D from SMS 60-RLV 40% reduced *IL1B* and *CXCL8*. These findings provide insights that both fungal species and substrate composition influence immunomodulatory compound production. Valorizing lignocellulosic residues through optimized mushroom cultivation represents a sustainable strategy for producing functional ingredients with applications in human and animal health, particularly for preventing inflammation-related disorders.

Keywords: agricultural waste; oyster mushrooms; INFOGEST; immunomodulation; anti-inflammatory genes

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1. Introduction

Agro-industrial residues represent a noteworthy global challenge, particularly in the context of sustainable waste management and circular economy strategies [1]. Also, one-third of the edible parts of food produced for human consumption are globally wasted [2]. In this framework, the utilization of non-wood forest products represents a key priority within FAO programs, while both research and industrial sectors are increasingly directing their interest toward mushroom cultivation and hydroponic farming systems as promising

strategies for the valorization of these underutilized materials [3,4]. Spent mushroom substrate (SMS) is considered an organic by-product with bioactive compounds and precursors, making it a promising candidate for sustainable valorization strategies within a circular bioeconomy framework [5]. Beyond the generation of SMS, hydroponic cultivation produces significant amounts of roots of leafy vegetables (RLV), which is classified as agro-industrial waste [6]. Their transformation into functional biomaterials via fungal bioprocesses provides a synergistic benefit by simultaneously mitigating waste generation and enabling the production of biologically valuable compounds [7,8]. Among agro-industrial waste, the fruit bodies, stipes or misshapen mushrooms mainly from the genera *Agaricus* and *Pleurotus* are promising substrates for the cultivation of new mushroom crops, which can effectively synthesize a broad spectrum of bioactive macromolecules [9,10]. Consequently, the valorization of these by-products for feed and/or food related applications would shift their status from waste to valuable resources within a circular production framework [11].

Among edible mushrooms, the production of *Pleurotus* spp. has shown a continuous increase in recent years, mainly due to their ability to utilize a variety of lignocellulosic biomass residues as substrates [12]. The genus *Pleurotus*, widely known as 'oyster mushrooms', comprises several species exhibiting numerous health-promoting benefits [13,14]. Many species within this genus, including *P. ostreatus*, have been extensively studied as sources of bioactive compounds, such as proteins, peptides and polysaccharides [10,15,16]. These compounds, present in both the mycelium and fruiting bodies of *P. ostreatus*, have been reported for immunomodulatory, antidiabetic, antiatherosclerotic, antitumor, anti-inflammatory, antibacterial and antioxidant activities [17,18]. Furthermore, it can be cultivated under relatively simple environmental conditions and shows strong tolerance to common diseases and pests [19]. These characteristics make its production advantageous, while its growth cycle is shorter than that of many other edible mushroom species [19]. Consequently, the cultivation of *P. ostreatus* represents a highly suitable alternative for mushroom production. One more species that has gained attention is *P. citrinopileatus*, commonly known as 'golden oyster mushroom', which is important due to its ability to grow on a wide range of lignocellulosic biomass substrates as well as its nutritional value [15,20–22]. The fruit bodies of *P. citrinopileatus* contain diverse bioactive molecules, including polysaccharides, proteins, phenolics and glucans, linked to health-promoting effects [23–26]. These traits, along with its established commercial production, point to *P. citrinopileatus* as a valuable and sustainable species for mushroom cultivation.

Both aforementioned *Pleurotus* mushrooms are commercially produced on substrates composed mainly of wheat straw [27]. Nonetheless, *Pleurotus* mushrooms can be cultivated on mixed lignocellulosic substrates, with the specific composition affecting cultivation performance [15,28,29]. In addition to their cultivation flexibility, *Pleurotus* species are rich in bioactive macromolecules, including valuable proteinaceous compounds as well as structurally complex polysaccharides, such as lectins and β-glucans, respectively [10,26,30,31]. Collectively, these fractions have been associated with immunomodulatory and anti-inflammatory effects, supporting the growing interest in *Pleurotus*-derived biomolecules for functional food and nutraceutical applications [32]. Therefore, agro-industrial wastes, including SMS and RLV, have attracted increasing interest for reuse in mushroom cultivation [3,10,15,16].

The biological activity of mushroom-derived macromolecules is widely recognized to depend on their structural characteristics. In particular, β-D-glucans are regarded as the most significant immunomodulatory polysaccharides among the various types of mushroom polysaccharides, associated with molecular weight, size, degree of branching, triple-helix conformation and solubility [33–35]. Similarly, the bioactivities of mushroom-derived

proteins and peptides are influenced by their primary sequences, three-dimensional structures, peptide sequences, amino acid composition, molecular mass distribution and susceptibility to enzymatic hydrolysis, microbial fermentation or gastrointestinal digestion [36–38]. Increasing evidence suggests that digestion-related structural modifications, such as partial degradation may influence the exposure of bioactive motifs and, consequently their activity [39,40].

In recent years, the integration of in vitro gastrointestinal digestion prior to cell culture systems has enabled the evaluation of interactions between food and feed components, such as proteins and carbohydrates and immune cells, including macrophages [41]. This approach provides a physiologically relevant representation of in vivo gastrointestinal conditions, minimizing ethical concerns, as well as enabling high-throughput experimentation under strictly controlled settings [42]. Consequently, it allows a reliable assessment of digestion-derived bioactive compounds and their potential immunomodulatory effects [38]. In this context, the THP-1 human monocytic cell line is differentiated into macrophage-like cells using phorbol 12-myristate 13-acetate (PMA) and subsequently stimulated with lipopolysaccharide (LPS) to investigate immunomodulatory effects under controlled pro-inflammatory conditions [43].

In this context, low-molecular-weight peptides derived from dietary proteins of animal, plant and fungal origin exhibit enhanced bioavailability and biological activity following gastrointestinal hydrolysis [37,44–46]. These peptides are encrypted within native protein sequences and are released upon digestion, enabling potential interaction with biological targets. Accordingly, the <3 kDa fraction was selected to focus on the peptide population most likely to retain bioactivity following gastrointestinal digestion and subsequent cellular exposure.

At the cellular level, innate immune responses play a pivotal role in the recognition of exogenous macromolecules, leading either to the direct elimination of invading microorganisms or to the induction of cytokine production that orchestrates downstream immune responses [47]. Exogenous macromolecules, including pathogen-associated molecular patterns (PAMPs), such as LPS, are recognized by pattern-recognition receptors (PRRs), particularly Toll-like receptors (TLRs), expressed on monocytes and macrophages [48,49]. TLRs, in turn, initiate intracellular signaling cascades, including the nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB) pathway, which ultimately leads to the transcriptional upregulation of immune-related genes, including the secretion of cytokines, chemokines and co-stimulatory molecules [50]. Among these, key pro-inflammatory cytokines, such as IL1B and TNF; anti-inflammatory cytokines, such as IL10; pleiotropic cytokines, such as IL6 and chemokines, such as CXCL8, are major mediators regulated by the NF-κB signaling pathway [51–54].

Mushroom-derived polysaccharides (e.g., β-glucans) are primarily recognized by the innate immune receptor Dectin-1 and can act synergistically with TLRs [55]. This interaction triggers NF-κB activation depending on the structural characteristics of polysaccharides [56]. Specifically, molecular weight and branching frequency affect the downstream cytokine responses [57]. Likewise, low-molecular-weight peptides generated during digestion may modulate the NF-κB signaling pathway [58,59]. These observations highlight the significance of digestion-derived macromolecules rather than native extracts alone.

Previous studies, including our own work, have demonstrated that the use of SMS and other agro-industrial residues as cultivation substrates can influence the biochemical composition of *Pleurotus* spp., including variations in protein content and carbohydrate-related fractions [16,26]. These findings indicate that substrate formulation plays a role in shaping the nutritional profile of mushroom fruiting bodies.

However, despite this evidence, limited information is available on how such substrate-dependent variations translate into the immunomodulatory activity of macromolecular fractions. Variations in substrate lignocellulosic composition can alter the availability of carbon and nitrogen sources during fungal growth, potentially influencing polysaccharide structure and protein composition within the fruit bodies. Moreover, the combined impact of sustainable substrate valorization strategies, simulated gastrointestinal digestion and the bioactivity of the digestion-derived macromolecules remains largely unexplored.

Bridging this gap, the present study examined how substrate composition (SMS/RLV ratio) and fungal species (*P. ostreatus* and *P. citrinopileatus*) influence the immunomodulatory activity of protein and polysaccharide fractions following standardized in vitro gastrointestinal digestion. To elucidate the underlying molecular mechanisms, a THP-1 macrophage model was employed, focusing on targeted gene expression analysis of the TLR–NF-κB–cytokine signaling axis, thereby providing mechanistic insight into the immune-related effects of *Pleurotus*-derived macromolecules.

2. Materials and Methods

2.1. Fungal Strzains and Substrates' Preparation

In the present study, *P. ostreatus* AMRL 138 and *P. citrinopileatus* AMRL 155, belonging to the fungal culture collection of the Laboratory of Edible Fungi/ITAP/Hellenic Agricultural Organization—Dimitra, were used. The fungi were maintained on Potato Dextrose Agar (PDA; Condalab, Madrid, Spain) at 4 ± 1 °C. Before each experiment, the fungi were re-cultivated on PDA plates and incubated at 26 ± 1 °C and 75% relative humidity until complete mycelial colonization. Subsequently, grain spawn was produced following the method described by Philippoussis et al. [60].

SMS and RLV, both obtained from the Greek agricultural company Manitus S.A., were mixed in various proportions. In certain formulations, wheat bran (WB) and soybean flour (SF) were added as nutritional supplements. Six substrate formulations were prepared on a dry weight (d.w.) basis and listed in Table 1.

Table 1. Composition of substrate formulations (% d.w. basis) used for the cultivation of *P. ostreatus* and *P. citrinopileatus*.

Substrate Variant	Substrate Synthesis	SMS (%)	RLV (%)	WB (%)	SF (%)	WS (%)
Variant 1	SMS 80%	80	-	15	5	-
Variant 2	SMS 80%-RLV 10%	80	10	5	5	-
Variant 3	SMS 80%-RLV 20%	80	20	-	-	-
Variant 4	SMS 60%-RLV 30%	60	30	5	5	-
Variant 5	SMS 60%-RLV 40%	60	40	-	-	-
Variant 6	WS 80%	-	-	15	5	80

Spent mushroom substrate (SMS), hydroponic roots of leafy vegetables (RLV), wheat bran (WB), soybean flour (SF), wheat straw (WS).

All substrates were supplemented with 1% (*w*/*w*) calcium carbonate ($CaCO_3$; SdS, Peypin, France) to adjust the pH to approximately 7. Polypropylene autoclavable bags (1 kg) were filled with each substrate formulation and sterilized twice at 121 ± 1 °C for 1 h (1.1 atm). After cooling, the substrates were inoculated with 3 – 5% (*w*/*w*, f.w. basis) grain spawn of *P. ostreatus* and *P. citrinopileatus* [16,26,60]. Incubation of bags was carried out in a growth chamber (ENTERLAB, mod. GROW-1300 HR, Terrassa, Spain) at 26 ± 1 °C in the dark and lasted 17 – 23 days. After complete substrate colonization, the bags were transferred to a fruiting room under controlled environmental conditions. Fruit body induction was performed at 20 ± 1 °C and 90 – 95% relative humidity (RH), while fructification took place at 17 ± 1 °C and 85% RH. During harvesting, the temperature was maintained at

17 ± 1 °C, relative humidity was adjusted to 95% and the air exchange rate was regulated to keep CO_2 levels below 1000 ppm. Illumination was provided at 800 lux for 12 h per day using fluorescent lamps. Harvested fruit bodies were frozen at −20 ± 1 °C, freeze-dried using a Heto LyoLab 3000 (Heto-Holten A/S, Lillerod, Denmark) and subsequently milled to a fine powder with an analytical mill (Janke & Kunkel, IKA-WERK, Staufen, Germany) for further analyses. To isolate and purify proteins, lipophilic compounds and polysaccharides from the mushrooms, sequential procedures including alkaline solubilization, isoelectric precipitation, ethanol extraction, ultrasonic cell disruption, Sevag purification, centrifugation and freeze-drying were performed as described by Diamantis et al. [26], until protein extracts (PEs) and carbohydrate extracts (CEs) were obtained.

2.2. In Vitro Digestion Assay and Digests' Fractionation

The in vitro digestion assay was performed according to a methodology previously described by our group [61]. The experimental procedure was conducted separately for protein extracts (PEs) and carbohydrate extracts (CEs). Each formulation was digested on three separate days using freshly prepared reagents, resulting in three independent digestion trials for each extract type [26]. In addition, three blank digestion trials were performed. Overall, 39 digestion trials were performed for PE and an equal number for CE. In brief, samples (5 mL) were rehydrated overnight at 4 °C, yielding final concentrations of 40 mg/mL PE and 2.25 mg/mL CE, after which INFOGEST static in vitro digestion protocol was applied [62]. Upon completion of digestion, PE and CE digests (PE-Ds and CE-Ds) were heat-treated (85 °C, 10 min), cooled, centrifuged (1200× *g*, 5 min) and filtered (0.22 µm sterile PVDF syringe filters; Merck Millipore, Burlington, MA, USA). Protein digestates (PE-Ds) were further fractionated using 3 kDa MWCO membranes to obtain the <3 kDa fraction (PE-D-P3) via ultrafiltration (Merck Millipore, Burlington, MA, USA). Blank digestates (BL-D) and their corresponding fraction (BL-D-P3) were prepared by replacing mushroom extracts with water and following the same digestion and fractionation protocols. Samples were stored at −20 °C until further analysis.

2.3. THP-1 Cell Culture, Viability and Quantification of Immune-Related Gene Expression

The THP-1 human monocytic leukemia cell line was maintained in a humidified incubator (37 °C, 5% CO_2). RPMI 1640 medium (Biosera, Cholet, France) was supplemented with 10% (*v*/*v*) fetal bovine serum (Gibco ThermoFisher Scientific, Waltham, MA, USA), 10 U/mL (1% *v*/*v*) L-glutamine (Biosera, Cholet, France), 1 mM (1% *v*/*v*) sodium pyruvate (Biosera, Cholet, France), 100 U/mL (1% *v*/*v*) penicillin (Biosera, Cholet, France), 100 µg/mL (1% *v*/*v*) streptomycin (Biosera, Cholet, France) and 100 µM (1% *v*/*v*) non-essential amino acids (Biosera, Cholet, France) prior propagation of THP-1 cells. Monocytes (2×10^6 cells/well; 2.5 mL/well) were seeded in 6-well plates and treated with PMA (100 ng/mL; Sigma-Aldrich, Saint Louis, MO, USA) for 48 h. Subsequently, the medium was discarded, and adherent cells were incubated in PMA-free supplemented medium for an additional 24 h. Following this resting period, macrophages were challenged to LPS (100 ng/mL; LPS from *E. coli* 011; Sigma-Aldrich, Saint Louis, MO, USA). and either PE-D-P3 (10% *v*/*v*) for 24 h or CE-D (15% *v*/*v*) for 6 h [26]. Incubation time with LPS was selected based on the expected kinetics of action of each fraction. A 24 h exposure was used for protein-derived fractions to assess transcriptional responses, whereas shorter incubation period were applied for polysaccharide-derived fractions [63–66]. In parallel, BL-D-P3 and BL-D were used, respectively. For each biological digestion replicate, cell treatments were performed in triplicate wells.

Following cell treatment, total RNA extraction, DNase digestion, reverse transcription and qPCR were performed according to the manufacturers' instructions (Nucleozol,

Macherey-Nagel, Düren, Germany; DNase treatment, New England Biolabs, Ipswich, MA, USA; PrimeScript RT reagent kit, Takara Bio, Shiga, Japan; FastGene IC Green 2X qPCR Universal Mix, Nippon Genetics, Tokyo, Japan; respectively). Primer sequences were designed by the authors and described in detail in previous studies [26,61].

2.4. Statistical Analysis

Results are expressed as means ± standard error of means (SEMs) of triplicate analyses for each treatment. Prior to statistical analysis, data were tested for normality (Kolmogorov-Smirnov test) and homogeneity of variance (Levene's test). Statistical analysis was performed using two-way analysis of variance (ANOVA), considering mushroom species and substrate composition as fixed factors, as well as their interaction. When significant main effects were observed, differences between levels were further evaluated using Duncan's post hoc test ($p < 0.05$). Statistical analyses were performed with SPSS for Windows, version 22.0.0 (SPSS Inc., Chicago, IL, USA) and graphs were generated using the GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA).

3. Results

To gain insight into the mechanisms by which edible mushroom extracts, specifically protein and carbohydrate extract, (PE and CE, respectively) from two *Pleurotus* species (*P. ostreatus* and *P. citrinopileatus*) exert immunomodulatory effects and influence the balance between pro- and/or anti-inflammatory cytokines, the immunomodulatory properties of digested *Pleurotus* extracts (PE-D-P3 and CE-D; respectively) were evaluated in THP-1 derived human macrophages following in vitro digestion of mushrooms. Additionally, gene expression levels were analyzed in LPS-challenged PMA-induced THP-1 derived macrophages treated with PE-D-P3 and CE-D obtained from *Pleurotus* spp. cultivated on different agri-food by-products, namely spent mushroom substrate and hydroponic leafy vegetable roots (SMS and RLV; respectively).

3.1. Main Effect of PE-D-P3 on mRNA Expression of Inflammation-Related Genes

Figure 1 demonstrates the results regarding the cytokine expression levels, including the pro-inflammatory cytokines as well as the anti-inflammatory cytokines, following exposure to PE-D-P3 in LPS-challenged PMA-induced THP-1 derived macrophages. Also, gene expression levels of transcription factors and Toll-like receptors were also evaluated. Treatment with PE-D-P3 resulted in significantly lower transcriptional levels of *TLR2* and *IL6* compared to BL-D-P3 (Figure 1c and 1f; respectively), regardless of mushroom species. Similarly reduced expression levels were also observed for the pro-inflammatory genes *IL1B* and *TNF* (Figure 1e and 1i; respectively), in both mushroom species when compared to BL-D-P3. Notably, a more pronounced reduction of these genes was observed in *P. citrinopileatus*. Furthermore, PE-D-P3 derived from *P. citrinopileatus* led to significantly lower expression of *NFKB1* and *RELA* compared to both *P. ostreatus* PE-D-P3 and BL-D-P3 (Figure 1a and 1b; respectively). *CXCL8* expression was significantly down-regulated in THP-1 cells treated with *P. citrinopileatus* PE-D-P3 compared to the corresponding *P. ostreatus* treatment, while no significant differences were observed relative to the BL-D-P3 counterpart (Figure 1g). In contrast, *IL10* expression was significantly down-regulated in cells treated with *P. ostreatus* PE-D-P3 compared to BL-D-P3, with no significant differences observed compared to *P. citrinopileatus* PE-D-P3 (Figure 1h). No statistically significant differences were observed among treatments regarding *TLR4* expression (Figure 1d).

Figure 2 presents the transcriptional levels of the same panel of inflammatory cytokines in THP-1-derived macrophages treated with PE-D-P3 obtained from *Pleurotus* spp. cultivated on agri-food by-product substrate, consisting of varying proportions of SMS and

RLV. No statistically significant differences were observed in the PE-D-P3 samples either between different substrates or compared to BL-D-P3-treated cells regarding *NFKB1*, *TLR4*, *CXCL8* and *IL10* expression (Figure 2a, 2d, 2g and 2h; respectively). However, PE-D-P3 treatments significantly altered the expression of several key pro-inflammatory genes, including *IL1B*, *IL6* and *TNF*. Specifically, *TNF* expression was significantly down-regulated in SMS 80%-RLV 20%, SMS 60%-RLV 30% and SMS 60%-RLV 40% -treated cells when compared to BL-D-P3 (Figure 2i). On the other hand, *IL1B* expression was significantly lower only in groups SMS 80% and SMS 80%-RLV 20% (Figure 2e). Results regarding the effect of PE-D-P3 show that *IL6* expression (Figure 2f) was significantly higher in the blank and WS groups compared to all other treatments. Moreover, groups supplemented with a higher proportion of RLV exhibited even lower *IL6* expression levels compared not only to the blank and WS controls but also to the substrate formulation containing only 80% SMS. As shown in Figure 2, *RELA* and *TLR2* expression (Figure 2b and 2c; respectively) was found to be lower in SMS 80%-RLV 20% -treated cells when compared to BL-D-P3 (blank digest). No decrease, however, was observed in the other SMS groups, with or without RLV, when compared to WS (control group).

3.2. Main Effect of CE-D on mRNA Expression of Inflammation-Related Genes

The effects of CE-D on cytokine gene expression were evaluated in LPS-challenged, PMA-differentiated THP-1 macrophages, focusing on both pro- and anti-inflammatory markers, as well as selected transcription factors and Toll-like receptors. Figure 3 shows the effects of the CE-D derived from different mushroom species, whereas Figure 4 depicts the effects of the cultivation substrate, irrespective of mushroom species. Specifically, *RELA* expression was significantly increased in both CE-D-treated cells when compared to BL-D (digestion with water instead of carbohydrate extract), irrespective of the mushroom's species (Figure 3b). In contrast, *TNF* expression was significantly lower in both CE-D-treated cells when compared to BL-D; however, no differences were observed between the two mushroom species (Figure 3i). No statistically significant differences were detected in the expression levels of *NFKB1*, *TLR2*, *TLR4*, *IL1B*, *IL6*, *CXCL8* and *IL10* between the CE-D-treated mushroom samples, regardless of species, nor when compared to BL-D-treated cells were observed (Figure 3a, 3c–3h; respectively).

NFKB1 expression, a key transcription factor of the NF-κB pathway, was significantly lower in the SMS 80% group compared to the WS, while no differences were observed among the other RLV-supplemented groups or BL-D (Figure 4a). Similarly, *RELA*, another major transcription factor of the NF-κB pathway, exhibited significantly reduced expression in the SMS 80% group compared to WS (Figure 4b). No significant differences were detected among the remaining RLV-supplemented groups; however, BL-D differed from WS, with higher *RELA* expression levels observed in the WS group (Figure 4b). In contrast, CE-D treatment significantly increased *IL1B* and *CXCL8* expression levels exclusively in the SMS 80% group compared to WS and to the SMS 60% groups supplemented with either 30% or 40% RLV (Figure 4e and 4g; respectively). No statistically significant differences in the expression of *TLR2*, *TLR4*, *IL6*, *IL10* and *TNF* between the CE-D-treated samples, regardless of substrate, or when compared to BL-D-treated cells were observed (Figure 4c, 4d, 4f, 4h and 4i; respectively).

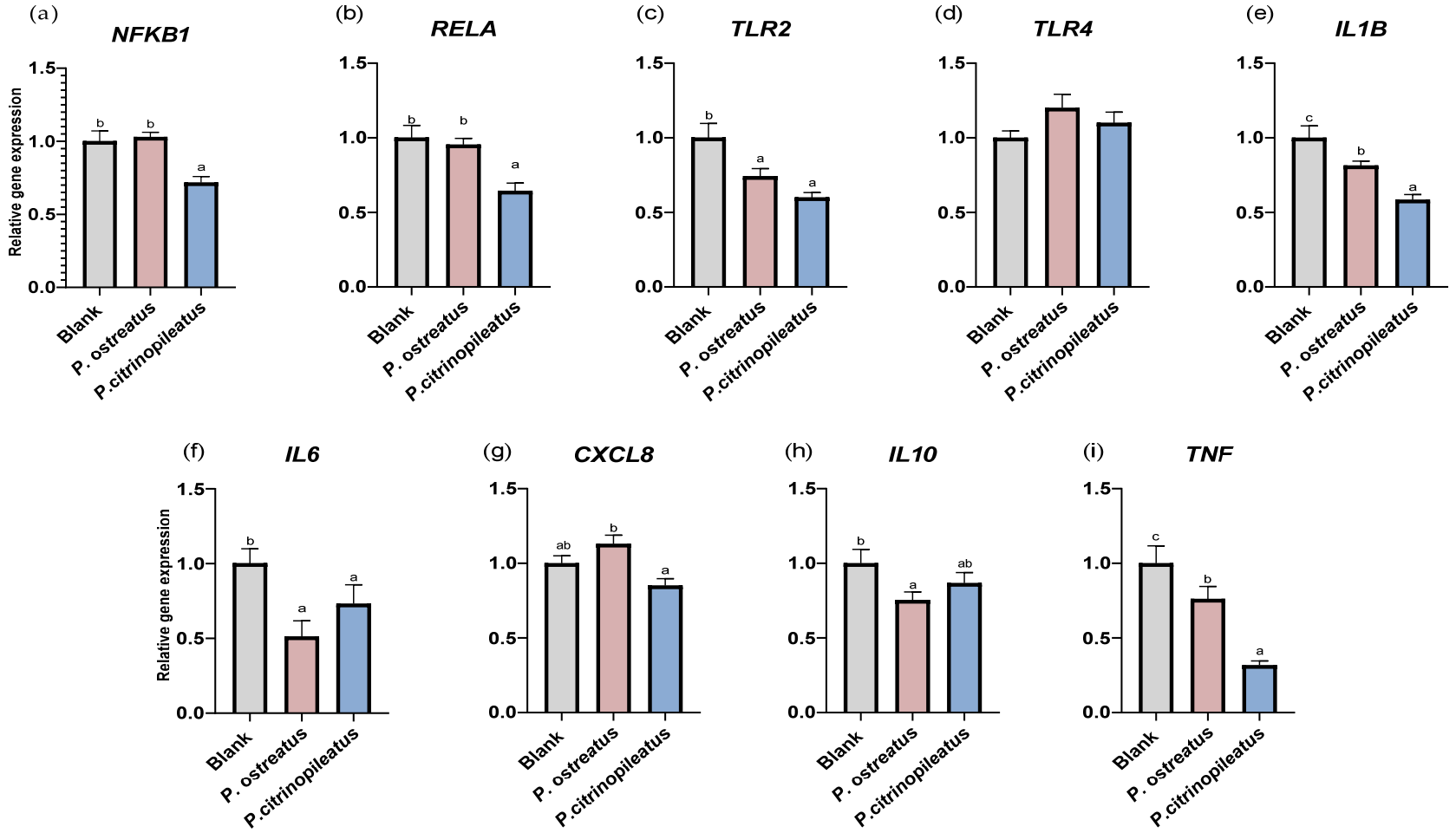


Figure 1. Main effect of mushroom species on gene expression in LPS-challenged PMA-induced THP-1-derived macrophages. Cells were treated with PE-D-P3 (4 mg protein/mL) derived from *P. ostreatus* and *P. citrinopileatus* or BL-D-P3 (Blank). mRNA expression levels of (**a**) *NFKB1*, (**b**) *RELA*, (**c**) *TLR2*, (**d**) *TLR4*, (**e**) *IL1B*, (**f**) *IL6*, (**g**) *CXCL8*, (**h**) *IL10* and (**i**) *TNF* were measured using qPCR. Data are represented as means ± SEM. Columns with different letters within the same panel are significantly different ($p < 0.05$).

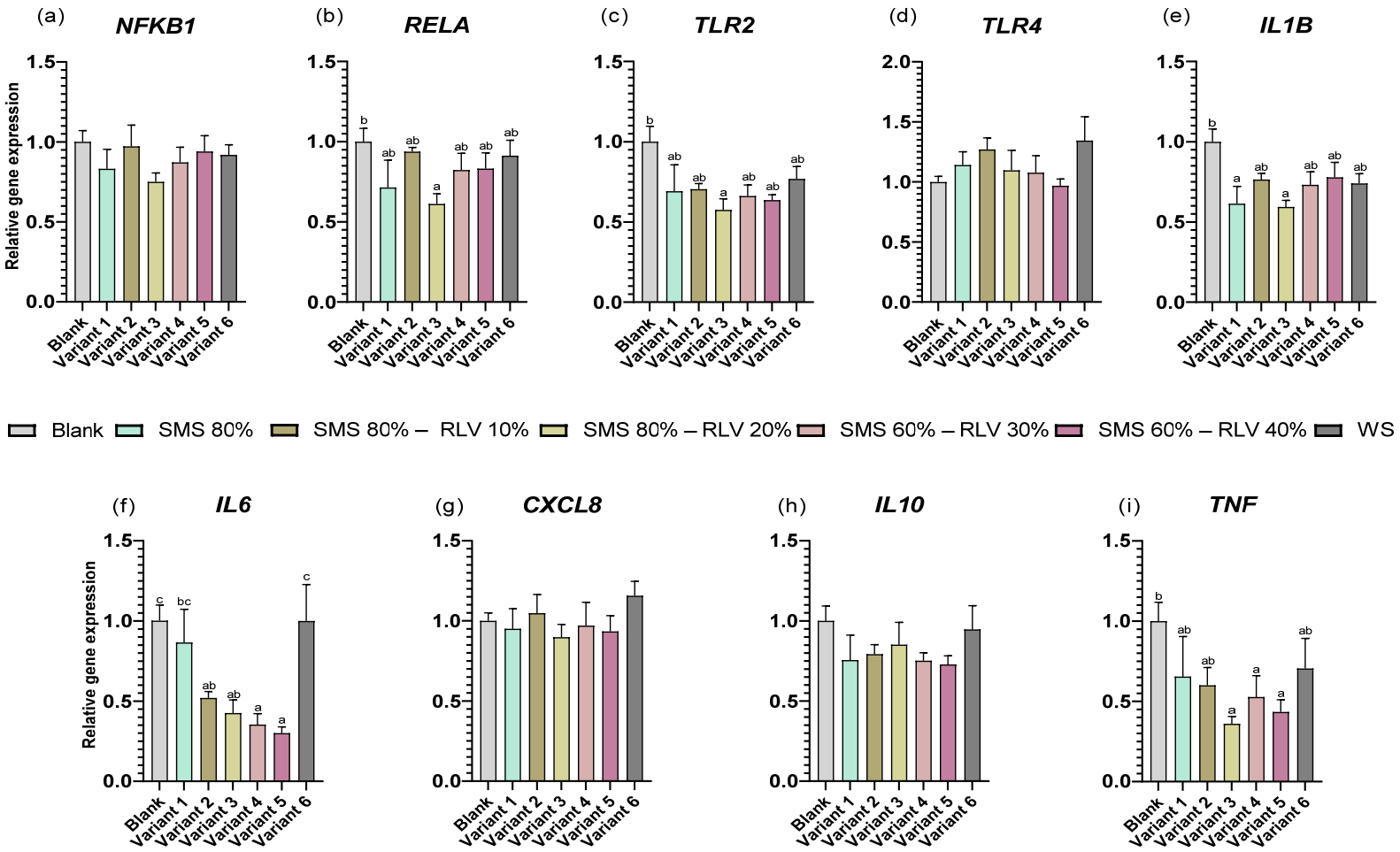


Figure 2. Main effect of substrate composition on gene expression in LPS-challenged PMA-induced THP-1-derived macrophages. Cells were treated with PE-D-P3 (4 mg protein/mL) derived from mushrooms cultivated on different agro-industrial residues (see Table 1) or BL-D-P3 (Blank). mRNA expression levels of (**a**) *NFKB1*, (**b**) *RELA*, (**c**) *TLR2*, (**d**) *TLR4*, (**e**) *IL1B*, (**f**) *IL6*, (**g**) *CXCL8*, (**h**), *IL10* and (**i**) *TNF* were measured using qPCR. Data are represented as means ± SEM. Columns with different letters within the same panel are significantly different ($p < 0.05$).

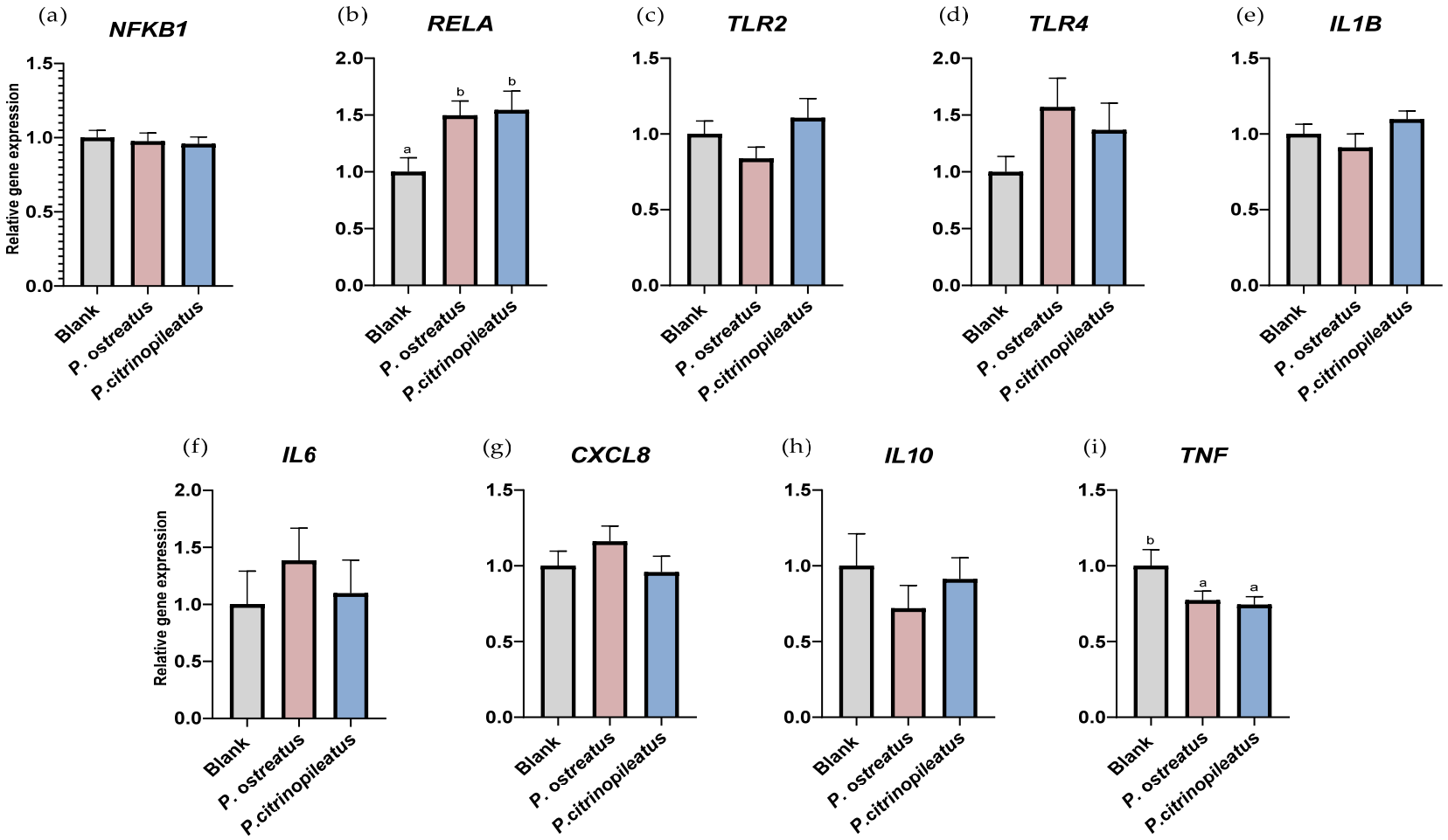


Figure 3. Main effect of mushroom species on gene expression in LPS-challenged PMA-induced THP-1-derived macrophages. Cells treated with CE-D (0.34 mg polysaccharides/mL) derived from *P. ostreatus* and *P. citrinopileatus* or BL-D (Blank). mRNA expression levels of (**a**) *NFKB1*, (**b**) *RELA*, (**c**) *TLR2*, (**d**) *TLR4*, (**e**) *IL1B*, (**f**) *IL6*, (**g**) *CXCL8*, (**h**), *IL10* and (**i**) *TNF* were measured using qPCR. Data are represented as means ± SEM. Columns with different letters within the same panel are significantly different ($p < 0.05$).

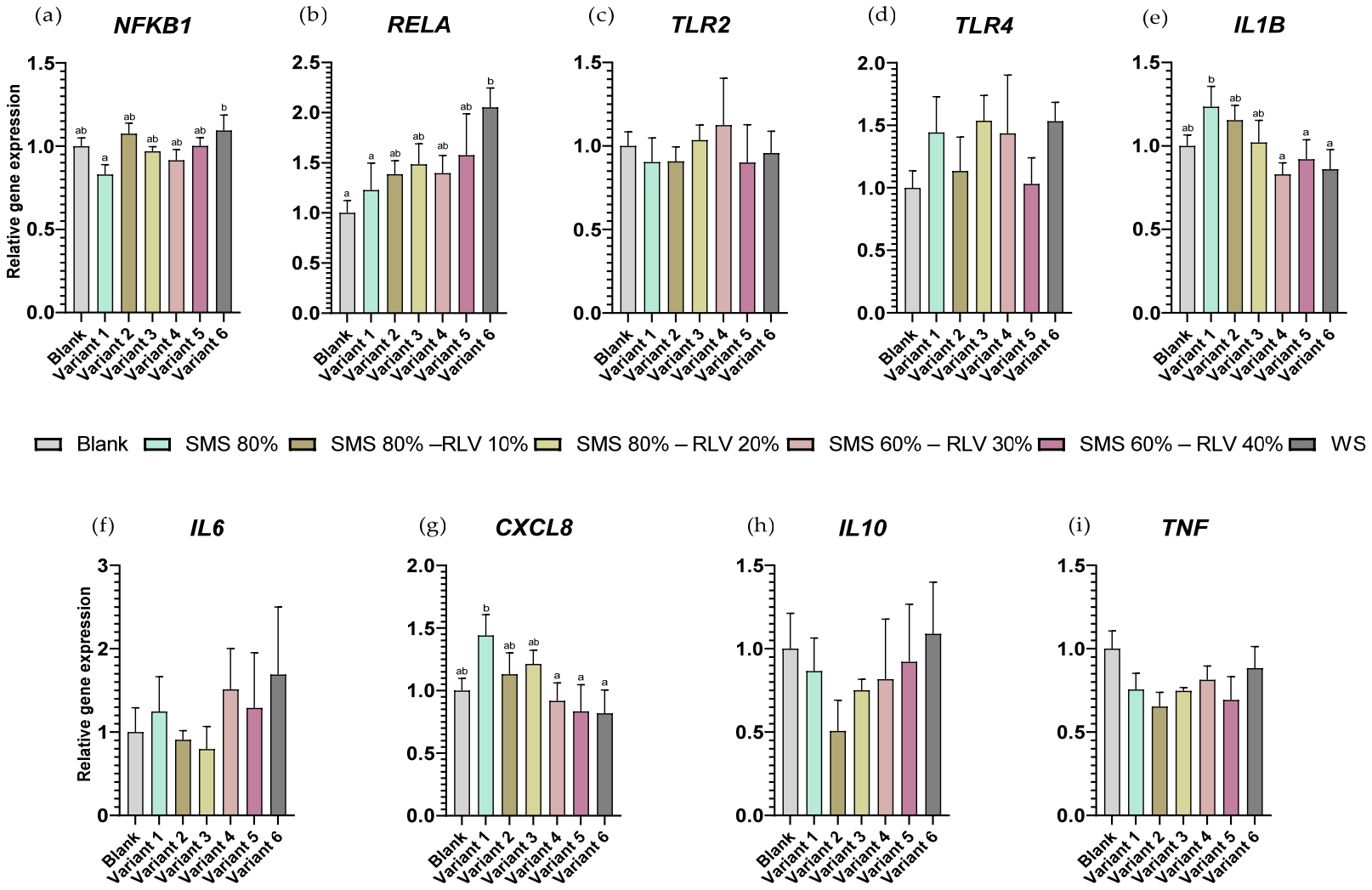


Figure 4. Main effect of the substrate composition on gene expression in LPS-challenged PMA-induced THP-1-derived macrophages. Cells were treated with CE-D (0.34 mg polysaccharides/mL) derived from mushrooms cultivated on different agro-industrial residues (see Table 1) or BL-D (Blank). mRNA expression levels of (**a**) *NFKB1*, (**b**) *RELA*, (**c**) *TLR2*, (**d**) *TLR4*, (**e**) *IL1B*, (**f**) *IL6*, (**g**) *CXCL8*, (**h**), *IL10* and (**i**) *TNF* were measured using qPCR. Data are represented as means ± SEM. Columns with different letters within the same panel are significantly different ($p < 0.05$).

3.3. Interaction Effect of Mushroom Species and Substrate Composition on Gene Expression in LPS-Challenged THP-1-Derived Macrophages

The interaction between mushroom species and substrate composition (species × substrate) on cytokine gene expression was assessed in LPS-challenged, PMA-differentiated THP-1 macrophages for both PE-D-P3 and CE-D fractions. Figure 5 includes only genes for which a significant species × substrate interaction was identified.

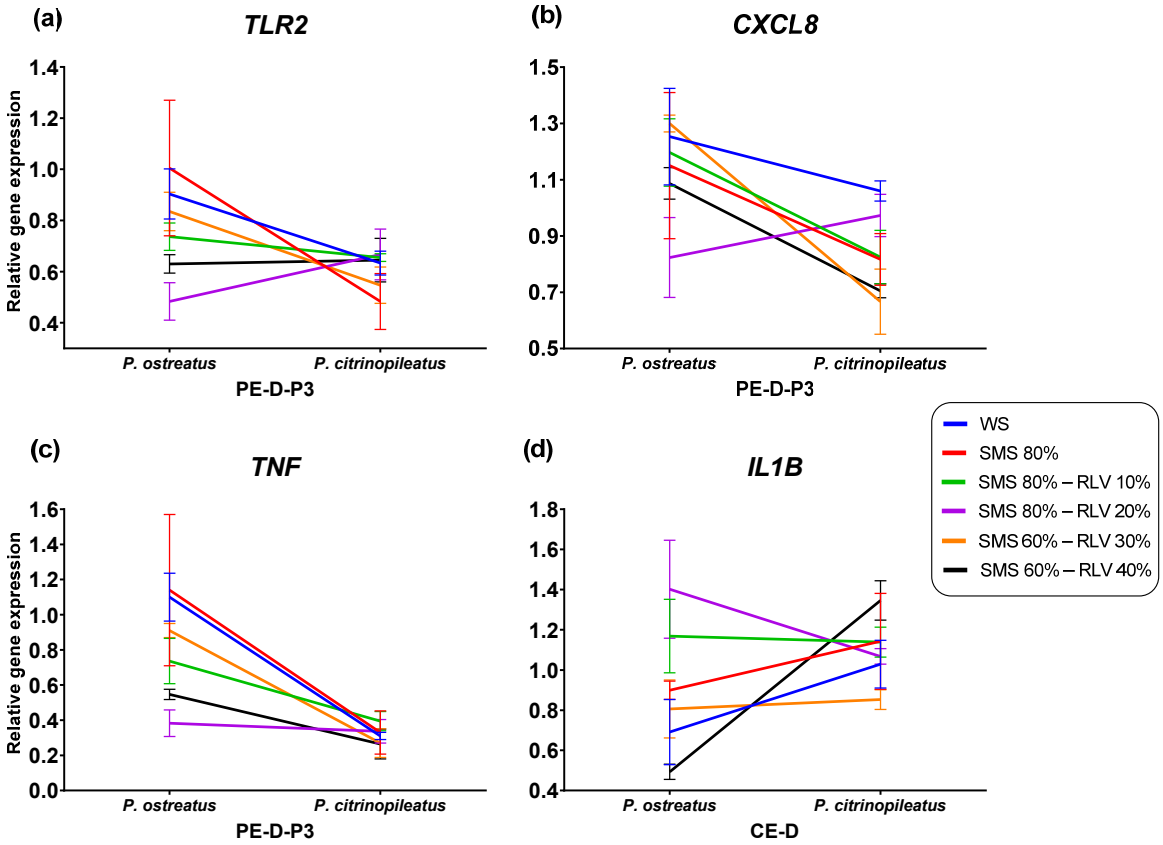


Figure 5. Interaction effect of mushroom species and substrate composition (species × substrate) on gene expression in LPS-challenged PMA-induced THP-1-derived macrophages treated with digestion-derived fractions. Cells were treated with (**a**–**c**) digestion-derived protein fraction (PE-D-P3) and (**d**) digestion-derived carbohydrate fraction (CE-D). Data of mRNA expression levels of (**a**) *TLR2*, (**b**) *CXCL8*, (**c**) *TNF*, and (**d**) *IL1B* are represented as means ± SEM. Interaction effects were evaluated using two-way ANOVA ($p < 0.05$).

For the PE-D-P3 fraction, the observed patterns indicate that gene expression responses differed between *P. ostreatus* and *P. citrinopileatus* depending on substrate composition, rather than following a uniform species-specific trend. For example, while *P. citrinopileatus*-derived PE-D-P3 generally resulted in lower expression levels for *TLR2* and *CXCL8* compared to *P. ostreatus*, this pattern was reversed under the SMS 80%-RLV 20% condition (Figure 5a,b). Similarly, for *TNF* expression, *P. ostreatus* tended to induce higher expression levels across most substrates; however, this difference was not observed under the SMS 80%-RLV 20% condition (Figure 5c).

For the CE-D fraction, a significant species × substrate interaction was observed for *IL1B* (Figure 5d), with the SMS 60%-RLV 40% substrate representing the most distinct condition, where *P. ostreatus*-derived CE-D exhibited approximately 3.5-fold lower expression compared to *P. citrinopileatus*.

Overall, these findings indicate that the immunomodulatory response of digestion-derived fractions is dependent on the specific combination of fungal species and substrate

composition, with each species exhibiting distinct response patterns across substrates, rather than consistent species-specific effects.

4. Discussion

Previous studies have demonstrated that mushroom-derived bioactive molecules can modulate the immune system, exhibiting antimicrobial, antidiabetic, hepatoprotective, antitumor and immunomodulatory properties, primarily attributed to structurally diverse macromolecules, including polysaccharides and protein-derived components [67]. Among edible fungi, species of the genus *Pleurotus* have been extensively investigated due to their richness in β-glucans, glycoproteins and bioactive peptides with pronounced immunomodulatory potential. In addition to species-specific characteristics, the use of agro-industrial residues as cultivation substrates represents a critical parameter influencing mushroom composition. Although substrate composition can affect mushroom biochemical profiles and functional properties [68,69], its impact on the immunomodulatory activity of mushroom-derived macromolecular fractions remains underexplored. To address this gap, the present study evaluated the immunomodulatory potential of protein and polysaccharide extracts from *P. ostreatus* and *P. citrinopileatus* cultivated under different SMS/RLV ratios, following simulated gastrointestinal digestion and assessment in THP-1-derived macrophages. This approach allowed investigation of how fungal species and substrate composition shape the biological activity of digestion-derived fractions, considering that gastrointestinal processing can substantially alter molecular structure and bioactivity.

Macrophages are known to respond more strongly than monocytes to mushroom-derived immunomodulatory compounds, including complete homogenates and polysaccharides, making PMA-differentiated THP-1 macrophages an appropriate model for evaluating cytokine responses to food-derived bioactives [70]. Supporting the role of mushroom-derived proteins in macrophage modulation, a polypeptide isolated from *Pleurotus eryngii* mycelium activated Ana-1 macrophages in a dose-dependent manner (0.2 – 1 mg/mL) without cytotoxicity, accompanied by increased TNF-α and IL-6 secretion and upregulation of *TLR2* and *TLR4* expression [71]. Lee et al. [72] reported that high-molecular-weight protein components from *Lignosus rhinocerotis* significantly inhibited LPS-induced TNF-α production in RAW264.7 macrophages, whereas the non-protein fraction showed no activity. These findings are consistent with our results, which highlight the contribution of mushroom-derived proteins to the modulation of macrophage-mediated inflammatory responses. In line with this, the KSPLY peptide was isolated and its biological activity assessed in LPS-stimulated RAW264.7 macrophages, confirming successful induction of M1 polarization [73]. In a follow-up study by the same group, the digested form of KSPLY significantly downregulated *TLR4*, *TNF*, *Il1B* and *Il6* expression, while increasing Il10 levels, indicating modulation through the TLR4/NF-κB signaling pathway [74].

The role of gastrointestinal digestion in shaping immunomodulatory activity has only recently begun to be clarified. Proteins and peptides from *Hericium erinaceus*, evaluated after in vitro digestion in a Caco-2/RAW264.7 co-culture model, demonstrated that lower molecular weight fractions exerted stronger immune-stimulating effects, confirming that peptide size is a critical factor of activity. These peptides significantly elevated NO production and enhanced TNF-α, IL-6 and IL-10 secretion compared with controls [75]. Such findings support the hypothesis that gastrointestinal digestion generates smaller, bioaccessible peptides that substantially contribute to the immunomodulatory potential of fungal proteins. The present study focuses on digestion-derived protein fractions, rather than on a molecular weight distribution analysis before and after digestion. Accordingly, the molecular weight distribution of the fractions was not characterized in the present study and could be further investigated in future studies.

Polysaccharides are generally considered more resistant to gastrointestinal enzymatic digestion compared to dietary proteins [76,77]; however, partial modifications in their molecular weight and structural features may still occur depending on their composition. Such digestion-related alterations may influence their biological activity and are closely linked with immunomodulatory effects. In the present study, these structural parameters were not directly characterized, therefore further analyses would be required to elucidate the relationship between digestion-induced modifications and bioactivity.

Regarding the immunomodulatory potential of polysaccharides, Mokhtar et al. [63] reported that isolated soluble *Pleurotus pulmonarius* polysaccharides induced a dose-dependent increase in *TNF*, *IL1B* and *IL6* expression in LPS-stimulated THP-1 macrophages. In contrast, in our study, *TNF* expression was significantly downregulated following CE-D treatment, whereas *IL1B* and *IL6* remained unchanged. Notably, methodological differences between the two studies may explain these discrepancies. These discrepancies may be attributed to methodological differences, including polysaccharide processing (isolated vs. digested fraction), concentration range (10 – 80 vs. 340 μg/mL), LPS dose (1000 ng/mL vs. 100 ng/mL) and exposure duration (24 and 48 h vs. 6 h.); all of which may differentially affect NF-κB activation and downstream cytokine responses.

Comparable attenuation of pro-inflammatory cytokines has been reported for a semi-purified polysaccharide extract (250 μg/mL) from *Agaricus brasiliensis* (syn. *A. blazei*), which significantly reduced *IL1B* and *TNF* production in LPS-stimulated THP-1-derived macrophages (100 ng/mL; 1 μg/mL LPS for 9 h) [65]. Although our study demonstrated *TNF* reduction without significant *IL1B* changes, differences in LPS concentration, extract processing (undigested vs. digested) and control comparisons (LPS vs. BL-D) may account for this divergence.

Similarly, no significant differences in *Il6* and *TNF* mRNA expression were observed in RAW264.7 macrophages (LPS: 5 μg/mL, 24 h; polysaccharides: 100 μg/mL) treated with polysaccharides *Ganoderma lucidum* and *Polyporus umbellatus* under inflammatory conditions, indicating that not all fungal polysaccharides exert anti-inflammatory effects and that activity depends on molecular structure and experimental context [78]. Additional evidence supporting polysaccharide-mediated regulation was provided by EL-Deeb, who demonstrated that crude *P. ostreatus* glucan extract reduced TNF-α levels in LPS/IL-2-induced peripheral blood mononuclear cells [79]. Furthermore, CE-D treatment in the present study resulted in reduced *TNF* expression compared to BL-D, supporting the regulatory capacity of mushroom-derived polysaccharides. However, polysaccharide bioactivity is highly structure-dependent, as molecular weight, branching degree and purity critically influence receptor interactions [57]. Murphy et al. [80] further demonstrated that purified β-glucan polysaccharides (1 mg/mL) from various mushroom species reduced of the inflammatory marker *CCL8* expression and increased *IL10* levels in LPS-stimulated THP-1 macrophages, suggesting an anti-inflammatory shift. In contrast, the digestion-derived polysaccharide fractions used in the present study likely represent heterogeneous mixtures with modified structural characteristics, which may account for the more selective cytokine modulation observed. Overall, these findings highlight the need for further structural investigations. Future studies incorporating detailed physicochemical characterisation before and after gastrointestinal digestion will be essential to fully elucidate the relationship between structural modifications and immunomodulatory potential.

Context-dependent effects of *Pleurotus*-derived preparations were also reported by Kerezoudi et al. [81], who observed no significant transcriptional changes in *NFKB1*, *TLR2*, *IL1B* and *IL10* in LPS-stimulated Caco-2 (100 ng/mL LPS for 24 h) cells treated with fermented supernatants (2% *v*/*v*) from the whole fruiting body of *Pleurotus eryngii*. Moreover, the intact food matrix exhibited a more diverse and synergistic bioactive profile than its

digested counterpart [81]. These observations align with our findings, where CE-D showed limited transcriptional modulation compared with PE-D-P3.

Beyond polysaccharides and proteins, other fungal extracts have demonstrated potent anti-inflammatory effects. Ethanol and aqueous extracts of *Xylaria nigripes* inhibited NO, PGE2, TNF-α, IL-1β and IL-6 production and suppressed NF-κB activation in LPS-stimulated peritoneal macrophage cells of Balb/c mice, with activity not directly correlated to total polyphenol content [82]. Fungal immunomodulatory proteins, approximately 13 kDa in size, have also emerged as promising bioactive molecules [83]. For example, recombinant protein from *Morchella conica* SH, defined as FIP-mco, significantly suppressed TNF-α, IL-1β and IL-6 expression in LPS-stimulated THP-1 cells via inhibition of NF-κB signaling [84].

The concept of immune modulation has also evolved, recognizing that balanced regulation of pro- and anti-inflammatory cytokines may promote immune homeostasis rather than complete suppression of inflammation [85]. Structural characteristics of polysaccharides derived from animal, plant and fungal sources are key determinants of macrophage activation patterns [86–88]. Mechanistically, fungal polysaccharides may activate innate immune responses via TLR4/NF-κB signaling, as shown for polysaccharides from *Craterellus cornucopioides* [89] and *Leccinum rugosiceps*, which induced NO, IL-6 and TNF-α secretion in RAW264.7 macrophages [90].

Importantly, the influence of cultivation substrates on immunomodulatory properties remains underexplored. The interaction effects observed in the present study further support this notion, suggesting that substrate-driven compositional changes may differentially modulate the bioactivity of mushroom-derived fractions depending on the fungal species. Previous studies have primarily focused on *Pleurotus* spp. cultivated on agro-industrial residues, reporting that they can enhance biological efficiency, increase phenolic content, boost antioxidant and anti-tumor activities, modulate the gut microbiome and support prebiotic potential. These effects have been observed both in vitro, using immune and intestinal cell models, and in vivo, in animal models and human studies, underscoring that substrate composition not only influences mushroom yield and bioactive compound accumulation but also their functional and nutraceutical properties [29,91–94].

A previous study comparing extracts from commercially cultivated and wheat straw-cultivated *Ganoderma lucidum* reported differential effects on cytokine production by monocyte-derived dendritic cells, including variations in IL-6, TNF and IL-10 levels [95]. Similarly, *Pleurotus eryngii* grown on substrates containing olive mill waste showed that substrate composition altered glucan profiles and modulated inflammatory responses, with glucans derived from the stalks being more effective than those from the pileus in reducing LPS-induced TNF-α production [96]. Additionally, extracts from *Pleurotus pulmonarius* stalk waste stimulated the expression of immune-related genes in red hybrid tilapia following LPS challenge, with upregulation of *il1b* and *tnfa* observed in the midgut, spleen and head kidney [97]. Likewise, fungal residue extracts, including agro-industrial by-products, promoted beneficial gut bacteria and reduced lipopolysaccharide-binding in RAW264.7 cells, indicating significant immunomodulatory activity [98]. These studies collectively indicate that substrate-driven alterations in bioactive compound profiles can influence immune outcomes. To the best of our knowledge, the present study is the first to investigate the immunomodulatory potential of digestion-derived protein and polysaccharide fractions from *Pleurotus* species cultivated on alternative agro-industrial residues. Our findings demonstrate that such substrates can yield bioactive fractions capable of modulating inflammatory gene expression, with RLV supplementation notably enhancing reduction of *IL1B* and *IL6* expression. Notably, the two-way ANOVA revealed significant species × substrate interaction effects for key inflammatory markers, indicating that the immunomodulatory response

cannot be attributed to either factor independently. Instead, the biological activity of the digestion-derived fractions depended on the specific combination of mushroom species and cultivation substrate. While certain substrate formulations, such as SMS 80%-RLV 20%, were associated with a more pronounced reduction of selected pro-inflammatory markers in *P. ostreatus*-derived protein fractions, these responses were gene-specific and varied according to the extract type. Therefore, the present in vitro findings do not allow for definitive recommendations regarding the optimal substrate for mushroom cultivation for consumption. Rather, substrate selection should be considered within a multifactorial framework, including yield performance and overall nutritional composition, in addition to bioactivity.

Overall, the present study underscores the immunomodulatory potential of peptides and polysaccharides derived from *Pleurotus* species under well-defined in vitro conditions; however, extrapolation of these effects to in vivo settings remains premature. The clear heterogeneity in experimental approaches, including differences in extraction procedures, digestion protocols, cell models and readout endpoints, further complicates direct cross-study comparisons and warrants cautious interpretation. Therefore, subsequent investigations employing advanced in vitro systems (e.g., co-culture and dynamic gastrointestinal models), alongside appropriately designed animal trials and human intervention studies, are required to elucidate bioaccessibility, bioavailability, absorption kinetics and physiological efficacy. Such evidence will be critical to verify the functional exploitation of agro-industrial residues as sustainable sources of bioactive macromolecules in food and feed applications.

5. Conclusions

This study demonstrates that digestion-derived protein and carbohydrate fractions from *P. ostreatus* and *P. citrinopileatus* cultivated on agro-industrial residues exert immunomodulatory effects in LPS-stimulated THP-1-derived macrophages. The PE-D-P3 fraction showed the strongest activity, significantly reducing the expression of key pro-inflammatory genes, including *IL1B*, *IL6* and *TNF*, indicating its potential to attenuate inflammatory responses. In contrast, digestion-derived carbohydrate fractions exhibited more selective effects, primarily modulating *TNF* expression. Substrate composition (SMS/RLV ratio) influenced the bioactivity of the resulting fractions, underscoring the influence of agro-industrial residue mixtures on the functional properties of mushroom-derived macromolecules. Overall, these findings support the sustainable cultivation of *Pleurotus* species on alternative substrates as a promising strategy to produce bioactive compounds. Importantly, substrate composition emerges as a tunable parameter modulating the immunomodulatory properties of digestion-derived fractions, rather than a determinant of universally enhanced activity, with potential applications as functional ingredients targeting inflammation-related conditions.

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Abbreviations

The following abbreviations are used in this manuscript:

B2M	Beta-2-Microglobulin
BL-D	Blank Digestate (digestion with water instead of sample)
BL-D-P3	Blank Digested fraction below 3 kDa (digestion with water instead of sample)
CE	Carbohydrate Extract
CXCL8	C-X-C Motif Chemokine Ligand 8
D-P3	Digested fraction below 3 kDa
d.w.	dry weight
f.w.	fresh weight
IL1B	Interleukin 1 Beta
IL6	Interleukin 6
IL10	Interleukin 10
LPS	Lipopolysaccharides from Escherichia coli O111:B4
MWCO	Molecular Weight Cut-Off
NF-κB	Nuclear Factor kappa-light-chain enhancer of activated B cells
NFKB1	Nuclear Factor Kappa B Subunit 1
PAMPs	Pathogen-associated Molecular Patterns
PDA	Potato Dextrose Agar
PE	Protein Extract
PRRs	Pattern-Recognition Receptors
PMA	Phorbol 12-myristate 13-acetate
qPCR	Quantitative Polymerase Chain Reaction
RELA	Nuclear factor Kappa-B p65 subunit
RH	Relative Humidity
RLV	Roots of Leafy Vegetables
RPL37A	Ribosomal Protein L37a
RPS18	Ribosomal Protein S18
SGID	Simulated Gastro Intestinal Digestion
SF	Soybean Flour
SMS	Spent Mushroom Substrate
TLR2	Toll Like Receptor 2
TLR4	Toll Like Receptor 4
TLRs	Toll-like receptors
TNF	Tumor Necrosis Factor
WB	Wheat Bran
WS	Wheat Straw

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