



β -Glucan extracts from the same edible shiitake mushroom *Lentinus edodes* produce differential in-vitro immunomodulatory and pulmonary cytoprotective effects — Implications for coronavirus disease (COVID-19) immunotherapies

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ABSTRACT

Coronavirus pneumonia is accompanied by rapid virus replication, where a large number of inflammatory cell infiltration and cytokine storm may lead to acute lung injury, acute respiratory distress syndrome (ARDS) and death. The uncontrolled release of pro-inflammatory cytokines, including interleukin (IL)-1 β and IL-6, is associated with ARDS. This constituted the first study to report on the variability in physicochemical properties of β -glucans extracts from the same edible mushroom *Lentinus edodes* on the reduction of these pro-inflammatory cytokines and oxidative stress. Specifically, the impact on the immunomodulatory and cytoprotective properties of our novel in 'house' (IH-Lentinan, IHL) and a commercial (Carbosynth-Lentinan, CL) Lentinan extract were investigated using in vitro models of lung injury and macrophage phagocytosis. CL comprised higher amounts of α -glucans and correspondingly less β -glucans. The two lentinan extracts demonstrated varying immunomodulatory activities. Both Lentinan extracts reduced cytokine-induced NF- κ B activation in human alveolar epithelial A549 cells, with the IHL extract proving more effective at lower doses. In contrast, in activated THP-1 derived macrophages, the CL extract more effectively attenuated pro-inflammatory cytokine production (TNF- α , IL-8, IL-2, IL-6, IL-22) as well as TGF- β and IL-10. The CL extract attenuated oxidative stress-induced early apoptosis, while the IHL extract attenuated late apoptosis. Our findings demonstrate significant physicochemical differences between Lentinan extracts, which produce differential in vitro immunomodulatory and pulmonary cytoprotective effects that may also have positive relevance to candidate COVID-19 therapeutics targeting cytokine storm.

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1. Introduction

The pandemic outbreak of coronavirus disease 2019 (COVID-19) is rapidly spreading globally (Zhang et al., 2020; Rowan and Laffey, 2020). Reports from China showed that about 20% of COVID-19 patients developed severe disease, resulting in a fatality of 4% (Zhang et al., 2020). A large part of COVID-19 patients in China experienced severe complications including acute respiratory distress syndrome (ARDS) requiring admission to intensive care unit (ICU) (Zhang et al., 2020). ARDS is a devastating condition of severe respiratory fail-

ure with 40% mortality and for which novel therapies are urgently needed (Rezoagli et al., 2017). It is characterized by widespread inflammation of the lungs, where extrapulmonary infections are a key aetiology of ARDS onset (Laffey and Matthay, 2017). The inflammatory insult results in lung parenchyma injury and activation of the immune system with an up-regulation of pro-inflammatory cytokines (Laffey and Matthay, 2017). The current international standard of intervention includes ventilatory management and organ support (Bellani et al., 2016). The innate immune system plays a pivotal role in the pathophysiology of ARDS (Chousterman et al., 2017).

COVID-19 causes an inflammatory or cytokine storm (CS) in the lungs with the excessive and uncontrolled release of pro-inflammatory cytokines, including interleukin (IL)-1 β and IL-6 (Conti et al., 2020). The binding of COVID-19 to the Toll-Like Receptor (TLR) causes the release of pro-IL-1 β which is cleaved by caspase-1, followed by the pro-

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duction of IL-1 β that is a mediator of lung inflammation, fever and fibrosis. Researchers have confirmed level of inflammatory factors in patients with COVID-19 including elevation of IL-6 in non-survival groups (Huang et al., 2020), as compared with that of the survivals. Therefore, how to block the CS and when to initiate anti-inflammation therapy is critical for reducing the death rate of COVID-19 (Channapanavar and Perlman, 2017; Chousterman et al., 2017). Suppression of pro-inflammatory IL-1 family members and IL-6 has been shown to have a therapeutic effect in many inflammatory diseases, including viral infections (Zhang et al., 2020).

There is an increasing interest in the medicinal use of mushrooms nutraceuticals that have been previously reported to exhibit wide-ranging activities including anti-inflammatory, anti-tumor and immune-modulating capabilities (Pelizon et al., 2005; Zheng et al., 2005; Akramiene et al., 2007; Kumar, 2015). β -Glucans are one of the main active components derived from mushrooms (Smith et al., 2002; Zhu et al., 2015). These are glucose polymers that are linked together through 1,3 linear β -glycosidic chains. Complexity and variation in the compound derive from side branching structures (Stone, 2009). β -glucans isolated from fungi commonly possess side branching at the 1,4 or 1,6 position (Kaur et al., 2020). Variance will also occur with chain length and many these variances are species-specific and dictate biological activity (Sullivan et al., 2006; Chaichian et al., 2020). Lentinans are a specific class of β -glucans extracted from the edible mushroom *Lentinus edodes*, and are composed of a β -(1–3)-glucose backbone with two (1–6)- β -glucose branches of each five glucose units (Sullivan et al., 2006; Kaur et al., 2020). There has been an increasing interest in their use for treating disease in animals and humans (Carballo et al., 2019; McCarty et al., 2020). Carty et al. (2020) recently described the potential role of β -glucan as a natural nutraceutical for boosting type 1 interferon response to RNA viruses such as influenza and coronavirus.

Putative use of β -glucans in mitigating lung infections correlates with findings from our recent in vivo studies to address ARDS (Masterson et al., 2019). However, there were significant challenges in identifying a reliable and repeatable source of β -glucans suitable for lung delivery as findings from screening of over 20 natural and commercial products screened revealed that they were unsuitable for lung delivery due to microbial contamination or exhibiting very low levels of β -glucan. We recently reported that purified β -glucans (Lentinan) from the Shiitake mushroom *Lentinus edodes*, obtained using our in-house novel extraction method can be used to reduce populations of clinical isolate *Klebsiella pneumoniae* harbouring multiple antibiotic resistances in an in vivo lung infection model (Masterson et al., 2019). We reported that administration of Lentinan shows potential for treating sepsis-induced lung injury as it effectively reduces bacterial load in arterial blood and BAL, reduces white cell count protein inflammation to the lungs, and improves lung physiological parameters. Evidence also showed that in-house Lentinan extracted supported vital pO₂ along with promoting lung cellular repair.

This constitutes the first study to compare a commercially sourced Lentinan extract from the edible mushroom *Lentinus edodes* (referred to as Carbosynth-Lentinan) to that of an in-house hot-water extract (IHL) of the same mushroom in order to evaluate immunomodulatory properties. These were characterized using an in vitro lung injury model with a focus on profiling components associated with cytokine storm. It is hypothesized that β -glucans derived from exotic mushrooms have the potential to alleviate the immune cascade in pathological conditions, such as ARDS that is experienced by COVID-19 patients.

2. Materials and methods

2.1. Materials

Commercial Lentinan (CL) was sourced from Carbosynth (FL33321, Compton, Berkshire, UK). Fruiting bodies of *Lentinus edodes* were purchased from Fancy Fungi (Ringaheen, Co. Wexford Ireland). IHL was extracted from the fruiting bodies using a novel process. A549 cells (used at passage 90) and THP-1 cells (used at passage 10) were obtained from the American Type Culture Collection, (ATCC, Rockville, MD, USA). Cells were cultured in RPMI-1640 (Sigma-Aldrich, St. Louis, MO, USA), supplemented with 10% fetal calf serum (Sigma-Aldrich), 1% penicillin G (100 U/mL) and streptomycin (100 μ g/mL) solution (Sigma-Aldrich) at 37 °C in 95% air/5% CO₂ environment. For differentiation into macrophages, THP-1 cells were treated with phorbol 12-myristate 13-acetate (PMA) (Peptrotech EC, London, UK) at a concentration of 100 ng/mL for 48 h.

2.2. Physicochemical characterization of β -glucan samples

2.2.1. Megazyme analysis

Extracts were analyzed for (1,3)-(1,6)- β -glucan content using the Megazyme yeast and mushroom kit (K-YBGL) (Megazyme Ltd., Bray, Co. Wicklow, Ireland). Assays were carried out according to manufacturer's instructions. Briefly, samples were milled, and placed in 12 M H₂SO₄ at -4 °C for 2 h to solubilize the glucans. The samples were then hydrolyzed in 2 M H₂SO₄ at 100 °C for 2 h. After incubation, any remaining glucan fragments were quantitatively hydrolyzed to glucose using a mixture of *exo*-1,3- β -glucanase and β -glucosidase which gives a measure of total glucan. The alpha glucan and sucrose content of the sample is determined by hydrolyzing specifically to D-glucose and D-fructose. Glucose was measured with amyloglucosidase and invertase using a glucose oxidase peroxidase GOPOD reagent. β -Glucan was determined by the difference in both measurements.

Attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR).

ATR-FTIR was carried out on a Perkin Elmer Spectrum fitted with a universal ATR sampling accessory. All data were recorded at ambient temperature, in the spectral range of 4000–650 cm⁻¹, utilizing a 16 scan per sample cycle and a fixed universal compression force of 80 N. Subsequent analysis was carried out using Spectrum software.

2.2.2. Scanning electron microscopy (SEM)

SEM was performed on a Mira SEM (Tescan Oxford Instruments, UK) using a range of magnifications to evaluate the surface morphology of the extracts using the function of secondary electrons. Samples were placed on an aluminum stub and were gold-coated using Baltec SCD 005 sputter coater (BAL-TEC GmbH, Chemnitz Germany) for 110 s at 0.1 mbar vacuum before observation.

2.2.3. Nuclear magnetic resonance (NMR)

¹H NMR spectra were obtained using an Agilent Technologies Ultra High Field (UHF) 800 MHz NMR system. Spectra were analyzed using ACD NMR software. Samples were prepared in deuterated water (D₂O).

2.3. Immunomodulatory properties of β -glucan samples from *Lentinus edodes*

2.3.1. Cell viability assays

Cells were seeded at a density of 4 × 10⁵ cells/well in a 96 well plate. After 24 h, cells were treated with varying concentrations of both CL and IHL. Cells were incubated with samples for a further 24 h. For MTT assay, media was aspirated and cells were treated with 10% 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)

in RPMI for 3.5 h at 37 °C in a humidified incubator with 5% CO₂. MTT and media were aspirated and formazan product was solubilized through the addition of 100 µL of DMSO per well. Solubilized product was quantitatively measured at 540 nm using a Synergy™ HT Multi-Mode Microplate Reader (BioTek, Winooski, USA). Results were expressed as percentage viability with respect to vehicle control.

2.3.2. Enzyme linked immunosorbent assay (ELISA)

Human DuoSet sandwich ELISA kit (RnD Systems MN, USA) was used to measure cytokine levels in the medium after β-glucan exposure. All ELISAs were performed according to manufacturer's instructions. Results were expressed either in pg/mL or in ng/mL.

2.3.3. Cell injury

Pulmonary alveolar type II A549 cells were seeded at a density of 4×10^5 cells/well in 96 well plates. After 24 h cells were injured with 1 ng/mL of IL-1β (Peprotech, Rocky Hill, NJ) in RPMI supplemented with 1% penicillin/streptomycin. THP-1 cells were seeded at a density of 4×10^5 cells/well in 96 well plates and 24 h later injured with 100 ng/mL LPS (Sigma) in RPMI supplemented with 1% penicillin/streptomycin. Cells were then treated with β-glucan samples for a further 24 h.

2.3.4. Luciferase assays

A549 cells stably transfected with NF-κB-luc reporter gene were obtained from the National University of Ireland Galway. These A549 cells were seeded at a density of 4×10^5 cells/well in 96 well plates. After 24 h, IL-1β and β-glucan samples were added to each well for 3.5 h at 37 °C in a humidified incubator with 5% CO₂. 100 µL of luciferase substrate (SolarGlow Molecutools, Dublin, Ireland) which includes lysis buffer, was added to each well. After brief agitation on an orbital shaker, luminescence was assessed in a Microplate Reader (BioTek, USA). Results were expressed as fold induction with respect to vehicle treated controls.

2.3.5. Phagocytosis assay

The Vybrant™ phagocytosis assay kit (Invitrogen, Carlsbad, CA) was used to measure the phagocytosis capacity of THP-1 cells after exposure to β-glucan samples. Samples were analyzed according to manufacturer's instructions. Phagocytic index was calculated and results were graphed as percentage phagocytic index with respect to vehicle treated controls.

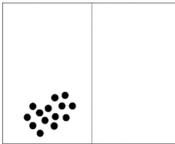
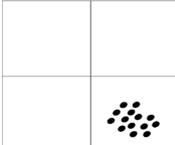
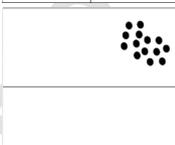
2.3.6. Hydrogen peroxide (H₂O₂) injury assay

To determine the effect of H₂O₂ on cell viability, cell apoptosis and necrosis were assessed using propidium iodide (PI) and Annexin-VFITC conjugated antibodies (Miltenyl Biotech). Cells were initially treated with a dose range of H₂O₂ from 0.001 mM to 20 mM. As 10 mM of H₂O₂, reduced cell viability by 50%, it was selected as the injuring concentration for further experiments. Cells were simultaneously treated with β-glucan samples. After 24 h of incubation, cells were complexed with Annexin-VFITC conjugated antibody at 1:1000 dilution for a further 10 min in the dark. Cells were washed in PBS three times and resuspended in flow buffer (Miltenyl Biotech). Cells were then automatically incubated with PI using the MACS Quant analyzer. Cells from each sample were then analyzed by MACSQUANT Analyzer 10 (Miltenyl Biotech). Data was analyzed using Flowlogic software (Miltenyl Biotech) with a guide to interpretation of findings represented in Table 1.

2.4. Statistical data analysis

Continuous data were described as mean ± standard deviation (SD). Differences in glucan content between CL and IHL were tested us-

Table 1
Interpretation of H₂O₂ assay derived from using flow-cytometry data.

Quadrant location:	Location representation:	Annexin V:	Propidium iodide:	Cell condition:
Lower left		Negative	Negative	Living cells
Lower right		Positive	Negative	Early apoptotic
Upper right		Negative	Positive	Late apoptotic cells
Upper left		Positive	Positive	Necrotic cells

ing unpaired Student's *t*-test. Differences of κB induction in A459 cells over increasing doses of CL and IHL were explored using a two-way analysis of variance. The viability of A549 cells was tested using increasing doses of β-glucans (i.e. IHL or CL) using a one-way analysis of variance. Post-hoc multiple comparison of increasing doses of CL and IHL versus 0 mg/mL was performed by controlling the false discovery rate using the two-stage step-up method of Benjamini, Krieger and Yekutieli. Differences in cytokine levels and phagocytosis were tested between different groups (i.e. LPS, CL and IHL) or between different doses (i.e. 1, 5 and 10 mg/mL) of β-glucans using a one-way analysis of variance. Post-hoc multiple comparison versus PBS was performed by controlling the false discovery rate using the two-stage step-up methods of Benjamini, Krieger and Yekutieli. Statistical significance was reached with a *p*-value < .05 (2-tailed). Statistical analyses were performed using GraphPad Prism 7a (GraphPad Software, San Diego, CA, USA) and Microsoft Excel for Mac 2017, Version 15.32 (Microsoft Corporation, Redmond, WA).

3. Results & discussion

3.1. Physicochemical characterization of β-glucan samples

Megazyme analysis of Carbosynth-lentinan (CL) and In-house Lentinan (IHL) samples are displayed in Fig. 1. Data shows both β and alpha glucan % w/w content. CL was shown to have a significantly higher alpha glucan content. IHL was shown to have a significantly higher β-glucan content and were purer and cleaner in composition. Scanning electron microscopy paired with energy dispersive X-ray analysis was carried out to determine particle size and elemental content, with results displayed in Figs. 2 and 3 respectively. Fig. 2, a dimensional representation of particle size, shows that CL has a uniform particle dispersity. Conversely, IHL shows a more heterogeneous particle mix. Fig. 3 shows that IHL contains fewer elements compared to CL, as sulfur, silicon and chlorine were

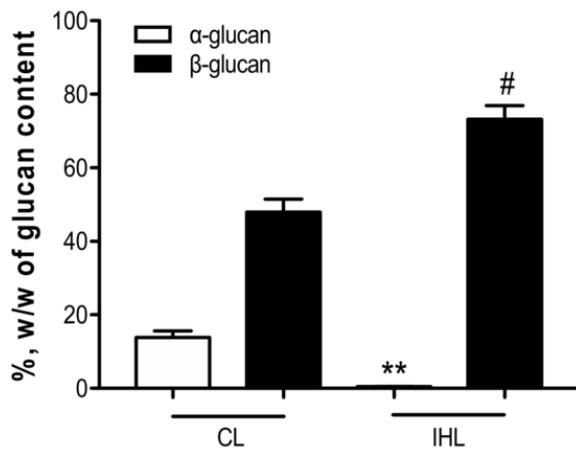


Fig. 1. Comparison of glucan content in commercial and in-house samples using Megazyme analysis. ** $p < .01$ versus alpha glucan C; # $p < .05$ versus β-glucan CL.

not present in IHL. These results, paired with Megazyme results, reaffirm that IHL is a cleaner preparation with fewer constituents.

FT-IR spectra of the CL and IHL are shown in Fig. 4. The IR analysis reveals a strong absorption peak in the fingerprint region at approximately 1015 cm^{-1} in both the commercial and in-house lentinan samples. The presence of equivalent absorption peaks, in the absorp-

tion range characteristic of polysaccharides, strongly suggests that both samples contain the same β-glucan compound. Preliminary NMR analysis of the commercial and in-house Lentinan suggests significant differences between the products. The ^1H spectra of the compounds are shown in Fig. 5.

Previous researchers have reported that variability in β-glucan chain length, branching and composition can vary based principally on source (Kaur et al., 2020). Extraction procedures will have an effect on the structure and purity of the β-glucan product, which may have an effect of its bioactivity. Thus, there has been an obstacle in understanding biological activities and this has hindered potential therapeutic development (Zhang et al., 2011). Scanning electron microscopy paired with energy dispersive X-ray analysis showed that the IHL had a diverse heterogeneous particle composition in comparison to CL, which had a more uniform composition. IHL was also found to have less chemical elements (sulfur, silicon and chlorine) compared to CL. These results suggest that IHL is a purer β-glucan product, compared to the commercially available sample. FTIR characterization of the samples shows that both samples contain the same β-glucan compound (Fig. 4). NMR analysis contradicted this and showed that there is a significant difference between both samples (refer to supplementary information). Bak and colleagues carried out a study to measure the glucan contents in the fruiting bodies of *L. edodes* mushroom from various cultivars which was found to be variable based on cultivar (Bak et

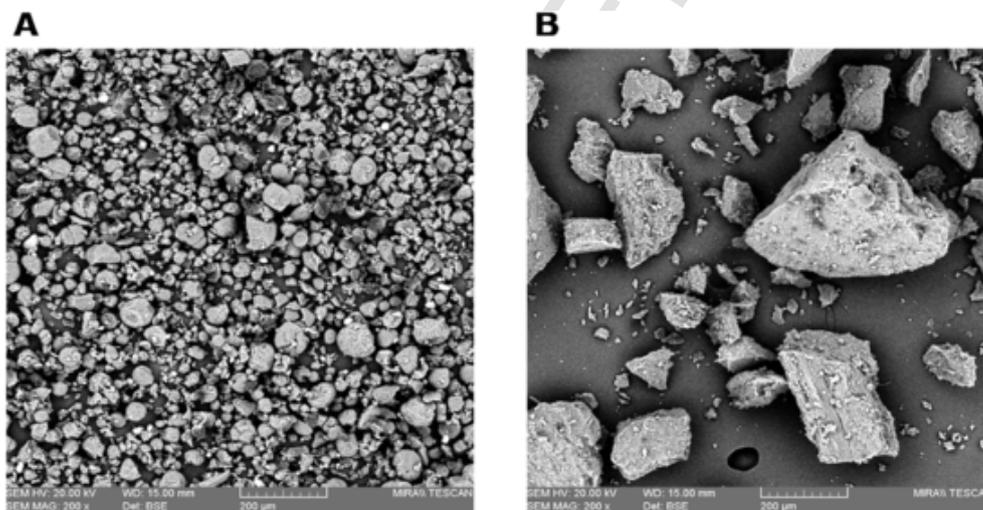


Fig. 2. Dimensional representation of commercial (A) and in-house (B) samples analyzed by scanning electron microscopy.

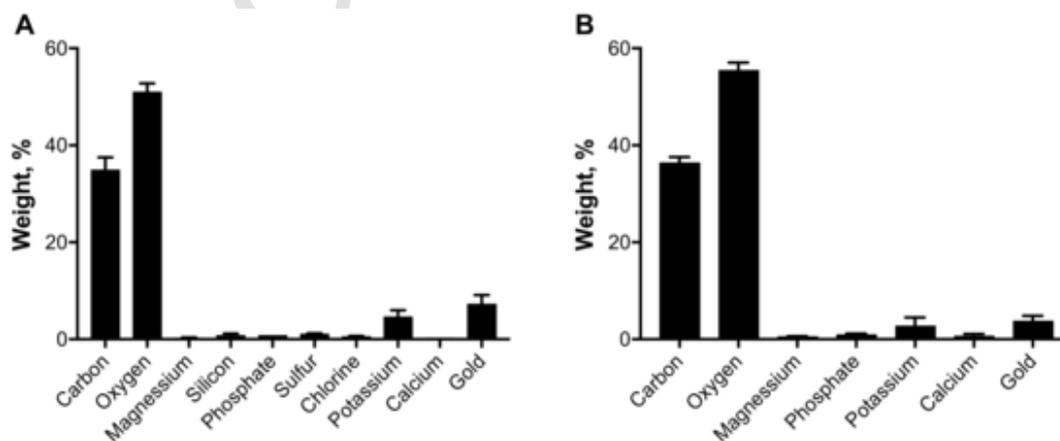


Fig. 3. Quantitative analysis of elemental contents in commercial (A) and in-house (B) samples determined by energy dispersive X-ray analyzer.

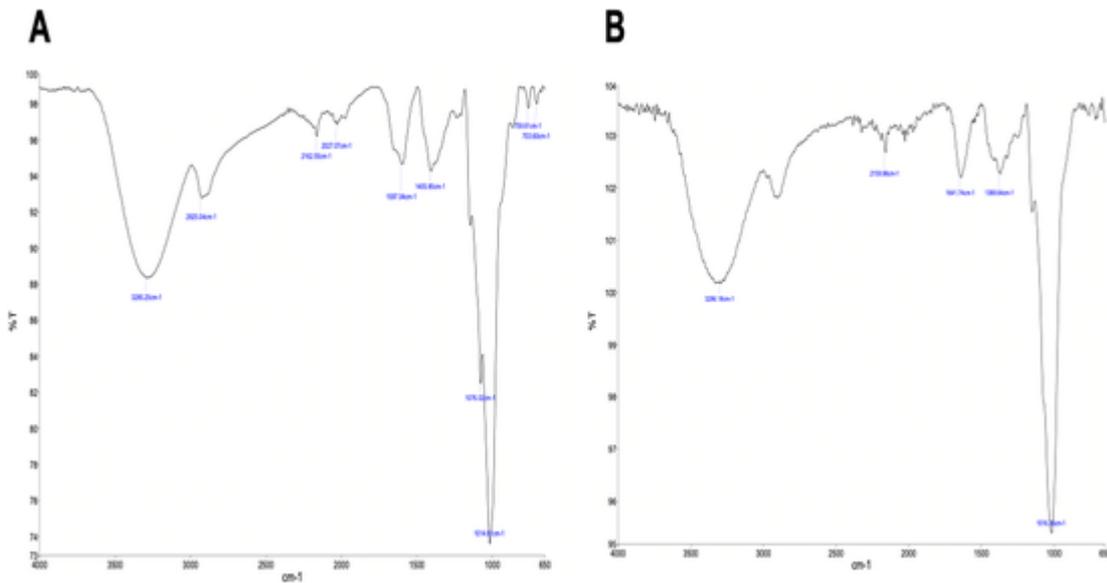


Fig. 4. FTIR spectra of the commercial (A) and in-house lentinan (B).

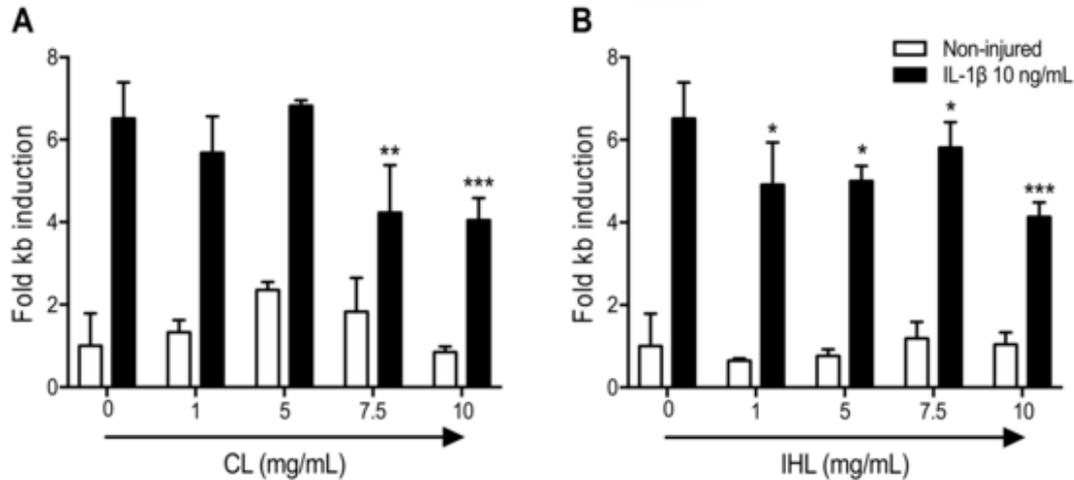


Fig. 5. A549 cells transfected with NF-κB reporter gene were stimulated with IL-1β for 1 h before administration of β-glucan samples. * $p < .05$; ** $p < .01$; *** $p < .001$ versus 0 mg/mL.

al., 2014). Gil-Ramirez and co-authors found a variance between mushroom samples based on growth conditions, degree of fruiting body maturing body as well as a difference between fresh and fruiting bodies (Gil-Ramirez et al., 2011). Studies have also shown variance in β-glucan content dependent on the country of origin (Bak et al., 2014). *L. edodes* from Japan (49.5% β-glucan w/w) having a higher content than that of mushrooms purchased in Iran (38% β-glucan w/w).

Immunomodulatory properties of β-glucan samples from Shiitake mushroom *L. edodes*.

To investigate the anti-inflammatory effects of the β-glucan samples, transformed human airway epithelial cells (A549) were used. Initial experiments showed that a dose of 10 ng/mL of IL-1β gave an IL-8 release response, indicative of NF-κB activation. To further examine the effects of the β-glucan samples on the NF-κB pathway, A549 cells stably transfected with the NF-κB luciferase reporter gene were used. IL-1β stimulation caused a 6-fold increase in luciferase expression. Both β-glucan samples reduced this expression, with IHL inhibiting this increase at a dose of 1 mg/mL (Fig. 5B). CL was able to reduce IL-1β induced NF-κB pathway activation at the higher concentrations (Fig. 5A). This IL-1β pulmonary model finding is directly relevant to COVID-19 as IL-1β is a prominent part of the 'cytokine storm' response (Zhang et al., 2020

). Hypercytokinaemia is considered a prominent mechanism of injury in COVID-19 patients. Furthermore, there are reports of the IL-1 Ra blocker Anakinra being effective for COVID-19, where Anakinra is being tested as a potential therapy for clinical trials. There are currently no treatments directed at halting the cytokine storm and acute lung injury to stop the progression from manageable hypoxia to frank respiratory failure and ARDS in patients with COVID-19 infection. Preventing progression from early acute hypoxia and cytokine release syndrome to frank hypoxic respiratory failure and ARDS could have a huge impact on the foreseeable overflow of the ICU units (<https://clinicaltrials.gov/ct2/show/NCT04330638>). The aforementioned reported that in ventilated patients, preventing the onset of ARDS, or shortening ICU stay could also be crucial in this regard. Furthermore, the clinical status after 15 days treatment was evaluated to measure the effectiveness of tocilizumab, tocilizumab and anakinra, siltuximab, siltuximab and anakinra and anakinra on restoring lung homeostasis, using single IV injection (siltuximab or tocilizumab) combined or not with daily subcutaneous injections of anakinra until 28 days or hospital discharge, whichever is first.

IHL was able to achieve these effects at the lower concentrations analyzed. MTT assays were performing to ensure that cell viability

was not contributing to the anti-inflammatory effects observed. Results show that both extracts did not elicit any cytotoxic effects at all concentrations, despite a trend toward a decrease in viability that was seen at the highest dose tested (10 mg/mL), but was only evident for the CL group ($p = .096$) (Fig. 6). No statistical differences were observed for multiple comparisons of single p -values for MTT findings, which is attributed to the broad data variability represented by large standard deviation observed (Fig. 6). Post-hoc multiple comparisons of increasing doses of CL and IHL versus test control (0 mg/mL) were used to ensure that MTT findings were not over-stated in the assessment of preclinical data to reduce risk of false discovery. Statistical analysis data of multiple comparisons for single p -values for MTT findings are also provided in supporting information.

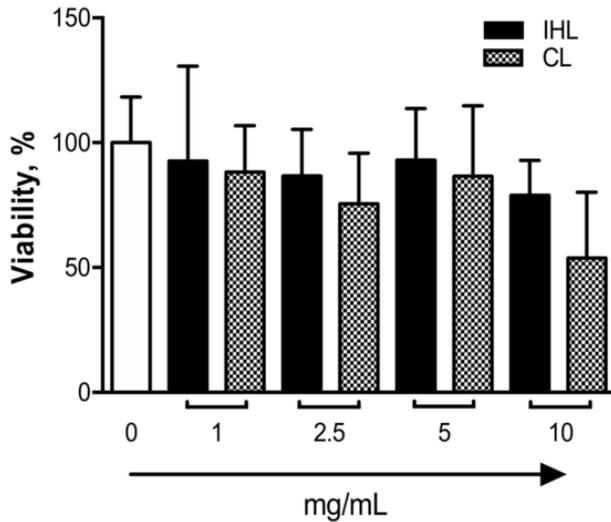


Fig. 6. MTT analysis of A549 cells treated with β -glucan samples to ensure anti-inflammatory properties are not related to a reduction in toxicity.

The capability of the β -glucan samples to induce or inhibit phagocytosis was also investigated. In the absence of injury, both CL (Fig. 7A+C) and IHL (Fig. 7B+C) appeared to reduce phagocytic index. After injury, both samples reduced phagocytic index (Fig. 7).

Macrophages and monocytes recognize β -glucans by various receptors present on their membrane (Vaclav et al., 2013). This recognition will result in the secretion of cytokines (Netea et al., 2008). Therefore, the immunomodulatory activity of the β -glucan samples was analyzed, in relation to their ability to influence the secretion of cytokines from macrophages. Results demonstrated that the samples induced/suppressed cytokine release at different concentrations. For example, IHL induced the secretion of IL-6 — conversely CL suppressed it. Lentinan analyzed by previous researchers was found to increase the release of TNF- α and IL-6 (Morales et al., 2019). This immunomodulatory effect was also analyzed by their ability to influence the phagocytic activity of macrophages. Both samples suppressed phagocytosis after LPS insult, but CL exclusively suppressed phagocytosis in the absence of injury. Dectin-1 receptor is assumed to be one of the main receptors responsible for the recognition of β -glucans. Activation of dectin-1 with 1,3-1,6 β -glucans can trigger cytokine release and phagocytosis (Brown and Gordon, 2001, 2005; Willment et al., 2003; Herre et al., 2004).

To determine the potential alleviating effects of the β -glucans on oxidative stress injury, THP-1 cells were treated with 10 mM of H₂O₂ to induce oxidative stress subsequent to β -glucan treatment. In terms of viability (Panel a), all doses of β -glucans increased viability after injury, CL however significantly increased viability at all doses administered (1, 2.5 and 5 mg/mL). CL significantly reduced early apoptosis after injury at all doses tested (Fig. 8B). IHL appeared to reduce both early and late apoptosis (Fig. 8A+B), however this was not significant. IHL at 1 mg/mL and 5 mg/mL, or CL at 1 mg/mL and 2.5 mg/mL appear to increase necrosis although not significantly (Fig. 8C).

Reactive oxygen species (ROS), which are generated as intermediates in metabolic pathways, are prevalent in pathological conditions. Oxidative stress can occur at a pulmonary level (Rahman and Mac-

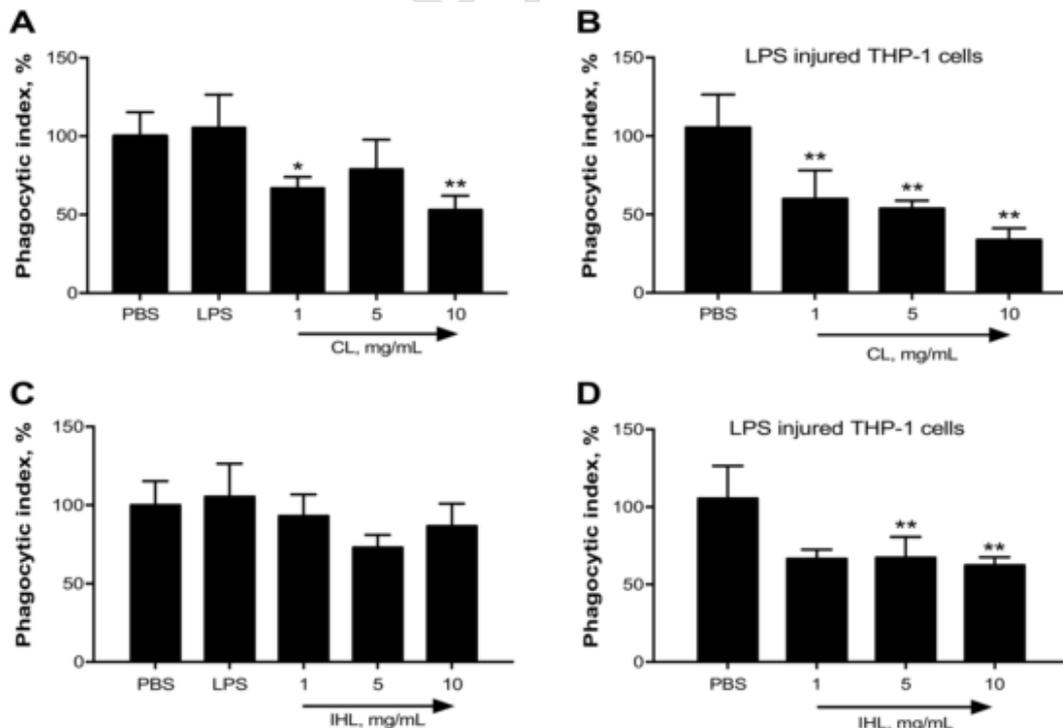


Fig. 7. The potential of β -glucan samples to alleviate phagocytic index of THP-1 cells was determined using Vybrant Phagocytosis assay kit * $p < .05$; ** $p < .01$; *** $p < .001$ versus PBS.

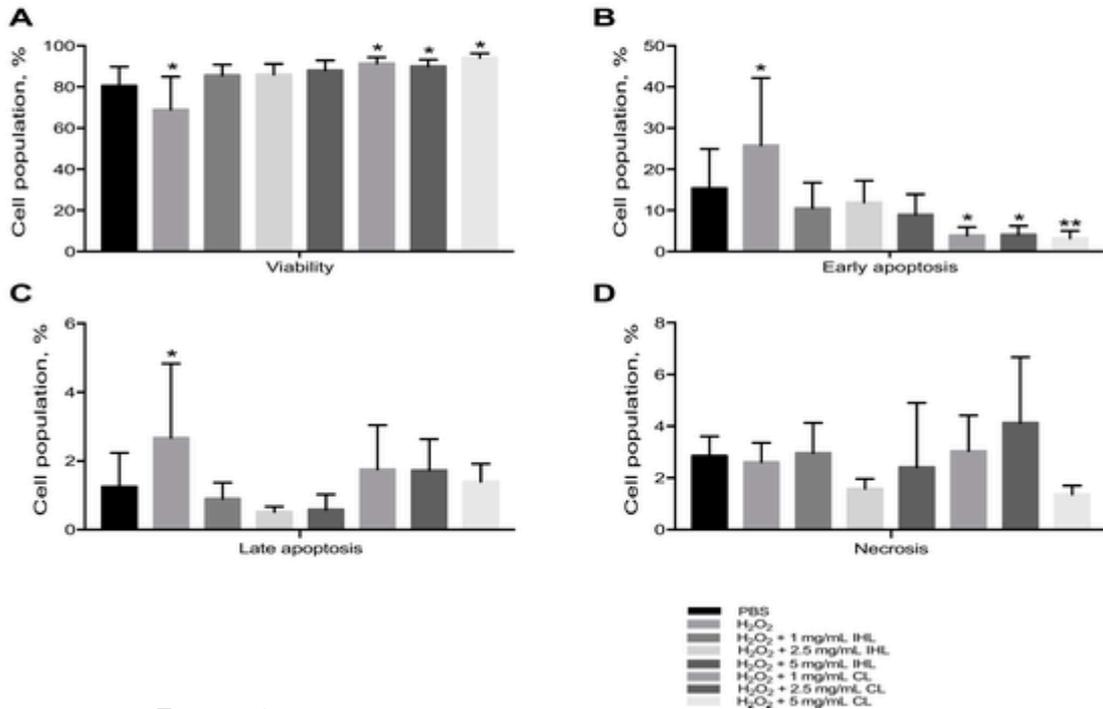
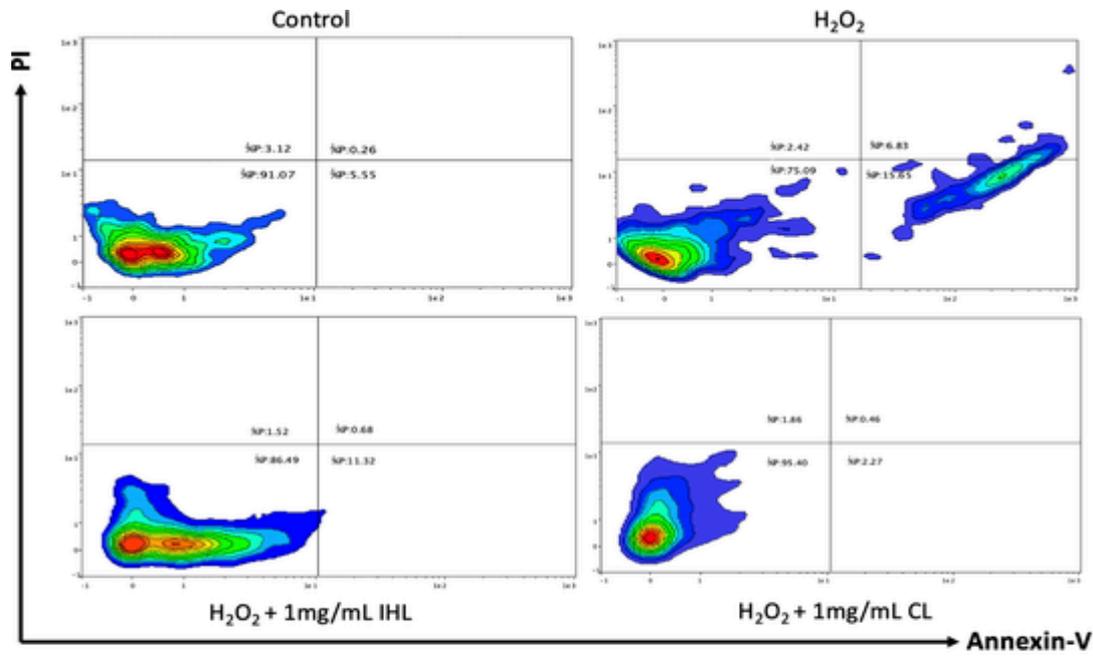


Fig. 8. Flow cytometric analysis of apoptosis (early and late) and necrosis of THP-1 cells after oxidative stress induction and subsequent β -glucan treatment * $p < .05$; ** $p < .01$; *** $p < .001$ versus 0 mg/mL.

Nee, 2000). Cellular-derived ROS are produced enzymatically by inflammatory and epithelial cells (Marwick et al., 2007). It has been suggested that oxidants play a contributing role to cell injury as well as leakage of fluid into the lung interstitial space (Liu, 2008). **The potential of β -glucan to alleviate oxidative stress is relatively unknown.** β -Glucans were used to investigate the ability to alleviate oxidative stress in H₂O₂-treated THP-1 cells where apoptosis and necrosis were measured using flow cytometry. Propidium iodide (PI) in conjunction with Annexin V was used to determine if THP-1 cells were viable, apoptotic or necrotic. Cell status was assessed based on differences in plasma membrane integrity and permeability (Vermees et al., 2000;

Farrell et al., 2011). The results from this experiment were variable and effects were source dependent. Both CL and IHL sources at both conditions tested had the ability to increase viability after injury. CL significantly reduced early apoptosis, and IHL showed a trend to reduce early apoptosis although not significantly. Our results are in line with the findings reported by Zi and colleagues who observed that Lentinan had the ability to alleviate oxidative stress (Zi et al., 2019). Reduction in oxidative stress by use of Lentinan is relevant to COVID-19 intervention. The renin-angiotensin (RAS) signaling pathway, oxidative stress and cell death, cytokines storm and endothelial dysfunction are four major pathways involved in the pathogenesis of COVID-19 (Kouh-

payeh et al., 2020). Therapeutic candidates that inhibit RAS and quench oxidative stress would be relevant for COVID-19.

An important assay to determine glucan effect is measurement of cytokine production. In this study, we investigated the effect β -glucan samples had on pro and anti-inflammatory cytokine expression. Inflammatory chemokines and cytokines measured included IL-8, IL-2 and TNF- α . Pleiotropic cytokines included TGF- β 1 and IL-6. Anti-inflammatory cytokines included IL-10 and IL-22 (Fig. 9). LPS (100 ng/mL) was also tested as it is a known inducer of inflammatory cytokines in immune cells. IHL (1 mg/mL) significantly induced the secretion of inflammatory mediators IL-8 and TNF- α . CL significantly induced the secretion of IL-8. IHL significantly increased the secretion of IL-10 and significantly decreased the secretion of IL-22, as did CL. Other cytokines measured included IL-6 and TGF- β 1. CL reduced the secretion of IL-6 and TGF- β 1. Conversely, IHL significantly increased the secretion of IL-6 and CL decreased secretion. No differences in TGF- β 1 concentration were observed when IHL was administered, however CL significantly reduced secretion.

In conclusion, findings of this in-vitro investigation showed that β -glucan from *Lentinus edodes* demonstrated potential for the treatment of lung injury. When compared to a commercial source of the same mushroom,

the in-house Lentinan extract contained higher levels of β -glucan and lower levels of α -glucan. Both Lentinan products reduced inflammation in a lung epithelial model, and IHL achieved this effect at lower doses. Physicochemical characterization studies found important differences in the composition of the Lentinan extracts, as determined by SEM, ATR-FTIR, and NMR, with the CL exhibiting higher amounts of alpha-glucans and correspondingly less β -glucans. The two Lentinan extracts demonstrated varying immunomodulatory activities. Both Lentinan extracts reduced cytokine induced NF- κ B activation in human alveolar epithelial A549 cells, with the IHL extract proving more effective at lower doses. In contrast, in activated THP-1 derived macrophages, the CL extract more effectively attenuated pro-inflammatory cytokine production (TNF- α , IL-8, IL-2, IL-6, IL-22). The CL extract attenuated oxidative stress-induced early apoptosis, while the IHL extract attenuated late apoptosis. The implications of these findings infer that defining a reliable and repeatable source of β -glucan, where processes can be tailored to control chain lengths may potentially reduce key cytokines involved in cytokine storm experienced in severe cases of COVID-19. In the future, β -glucans may be delivered as a tailored cocktail matched against critical time-points in the form of future nutraceutical-based intervention for tackling COVID-19. Such β -glucans im-

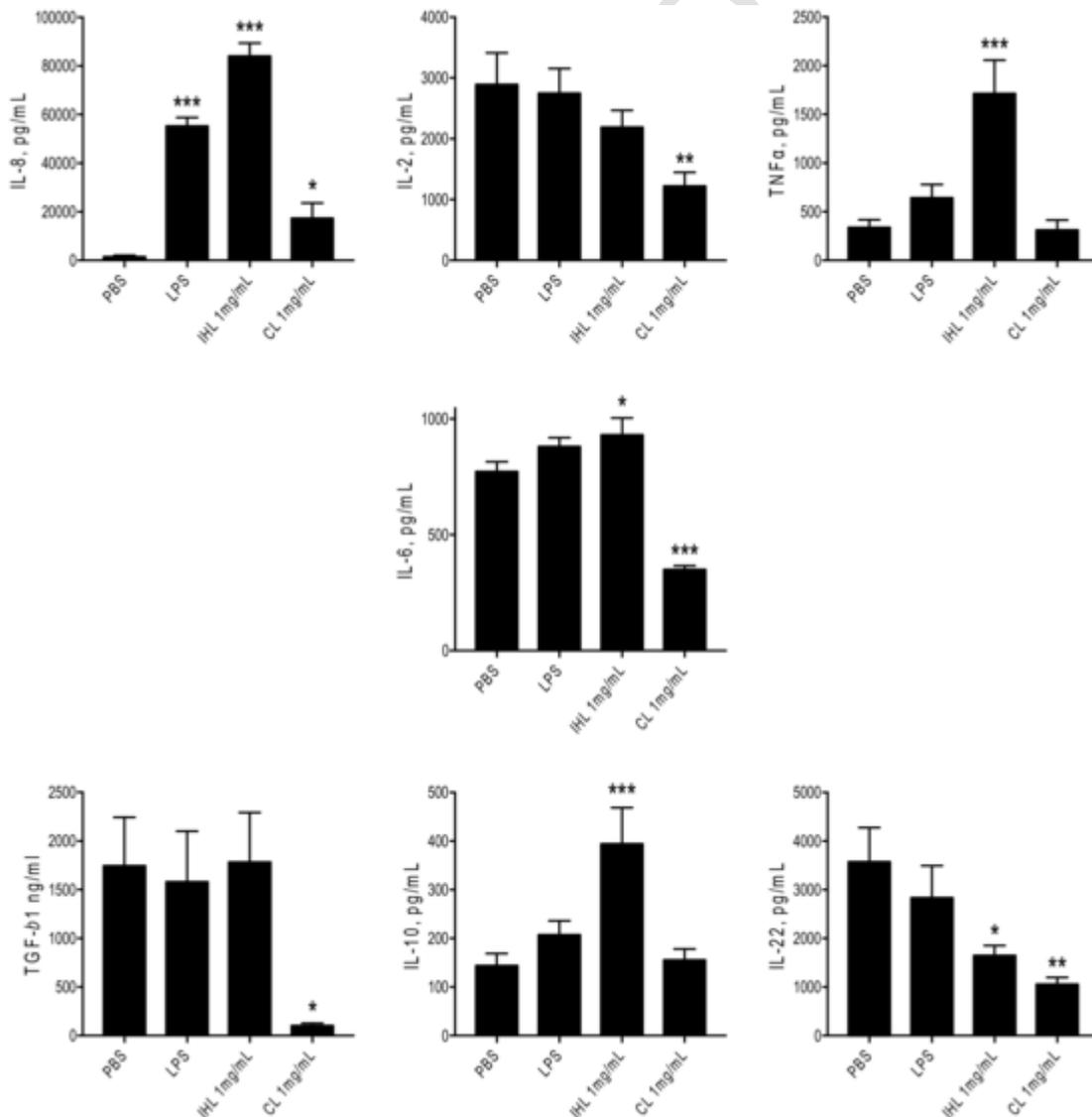


Fig. 9. The effects of β -glucan samples on cytokine expression of THP-1 cells preactivated with PMA. * $p < .05$; ** $p < .01$; *** $p < .001$ versus PBS.

munomodulatory cocktails may also have adjacent applications for addressing ARDS that is an important event seen with sepsis. These purified β -glucan combinational cocktails may be produced on a large commercial scale using bioreactors for global deployment (Tafuek et al., 2020).

To maintain functional bioactivity and to increase β -glucan yield, less harmful extraction processes are required that includes cessation of enzyme and harsh chemical usage as adopted using this IHL approach. Findings from this timely study highlight the putative potential for use β -glucan extractions from the edible mushroom Future research is also required to study putative relationship (if any) of variation in the extraction methods producing different β -glucan preparations and cytokine storm associated with COVID-19. *L. edodes* may also have future potential in the treatment of lung tissue repair along with informing immunotherapies for addressing COVID-19 that rely on reducing cytokine storm. Further clinical studies are merited to refine β -glucan as a countermeasure for tackling cytokine storm that causes ARDS, as evident with COVID-19.

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Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scitotenv.2020.139330>.

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