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## Article

# Optimization of Se- and Zn-Enriched Mycelium of *Lentinula edodes* (Berk.) Pegler as a Dietary Supplement with Immunostimulatory Activity

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**Abstract:** Mycelial cultures of *Lentinula edodes*, an edible and medicinal mushroom, have been used in our previous research to obtain selenium-containing immunomodulatory preparations. Our current attempts to obtain a new preparation containing both selenium and zinc, two micronutrients necessary for the functioning of the immune system, extended our interest in the simultaneous accumulation of these elements by mycelia growing in media enriched with selenite and zinc(II) ions. Subsequently, we have studied the effects of new *L. edodes* mycelium water extracts with different proportions of selenium and zinc concentrations on activation of T cell fraction of human peripheral blood mononuclear cells (PBMCs). Flow cytometry analysis was used to measure the expression of activation markers on human CD4+ and CD8+ T cells stimulated by anti-CD3 and anti-CD3/CD28 antibodies (Abs). It was demonstrated that all extracts with different proportions of polysaccharides, selenium and zinc tended to upregulate the expression of all activation markers on both types of T cell populations stimulated in parallel with anti-CD3/anti-CD28 beads, however, statistically significant changes were observed for PD-1 and CD25 antigens on CD8+ T cells. The selenium and zinc content in the examined preparations modified the immunomodulatory activity of mycelial polysaccharides, however, the mechanisms of action of various active ingredients of the mycelial extracts seem to be different.

**Keywords:** zinc–selenite interactions; polysaccharides; *L. edodes*; immunomodulation

## 1. Introduction

The global coronavirus pandemic has put pressure on researchers to develop, test, and deploy antiviral medications. Apart from medicines that act directly on the viruses, immune-stimulating preparations also seem to be interesting in the context of treating and preventing viral infections. Numerous reports on the immunomodulatory and antiviral activity of preparations containing zinc and selenium show that supplementation with these micronutrients may be beneficial for both prevention and treatment of viral infections, including coronaviruses [1]. Dietary multi-micronutrient supplements containing selenium up to 200 µg/day have potential as safe, inexpensive, and widely available adjuvant therapy in viral infections, e.g., Human Immunodeficiency Virus

(HIV) and Influenza A Virus (IAV). They can also be used in case of coinfections by HIV and Mycobacterium tuberculosis to support the chemotherapy and to improve the quality of life [2–6].

Zinc status, like selenium, is a critical factor that can influence antiviral immunity; zinc-deficient populations are often most at risk of acquiring viral infections such as HIV or hepatitis C virus [7,8].

The biological availability of zinc and selenium from the pharmaceutical preparations is limited by many factors, intrinsic and extrinsic to the host. For both micronutrients the bioavailability, tissue distribution, and toxicity strongly depend on the form ingested. In general, organic forms have a higher bioavailability and/or lower toxicity than inorganic species [9–11]. While both elements, zinc and selenium play a well-documented role in cancer prevention (e.g., for prostate cancer) and adjuvant therapy in viral infections (e.g., HIV, IAV), their combined supplementation is often given as a recommended prophylactic agent [12,13].

An interesting hypothesis, verified by the researchers for several years is synergism of the immunomodulatory effect of selenium (probably also zinc) and polysaccharides - mostly  $\beta$ -glucans of natural origin. Several members of the Basidiomycota division, often referred to as the “medicinal mushrooms”, can produce immunomodulating substances, which are usually polysaccharides [14–17]. Fungal polysaccharides are used as a non-invasive form of cancer treatment due to their ability to induce a non-specific response of the immune system against cancer cells. Further, this capacity is also evident against viral and bacterial infections and inflammation [18]. *Lentinula edodes* is among the most well-studied fungi capable of biosynthesis of the most active immunostimulatory polysaccharides (e.g., lentinan), used in several countries in the treatment of cancer, HIV, hepatitis and other diseases related to immunodeficiency. Further, fungi are unique in the accumulation of trace elements, which is a focus of interest of several research groups [19–24]. Thus, the submerged cultures of *Lentinula edodes* have been used in our previous research to obtain new immunomodulatory preparations enriched in selenium. We assumed obtaining a synergistic effect of polysaccharide compounds and selenium, which was confirmed in the already published studies [25–27].

In continuation, we have currently designed a new preparation containing immune-active polysaccharides and both selenium and zinc, two micronutrients necessary for the functioning of the immune system. The necessity to design a biotechnological process of cultivation the *L. edodes* mycelial cultures enriched in Zn and Se has extended our interest in the interactions between selenite and zinc (II) ions in the substrate and their effects on the transport of these ions into the fungal cell. In fungi, the process of accumulation of ions from solutions consists of three phases: biosorption on the surface of the fungal cell wall, intracellular uptake, and chemical transformation [28,29]. For the examination of the last two processes, living fungal biomass is required. Culture media contaminated with heavy metals usually have a very complex composition, with the result that metals biosorption may be affected by the presence of other ions. Moreover, the mechanisms of intracellular uptake and chemical transformation differ for cations and anions. For zinc(II) ions and selenites, investigated in this work, the mechanisms of accumulation by the fungus are also diverse and in higher mushrooms remain poorly understood. An examination of the mechanisms of zinc(II) transport in *Saccharomyces cerevisiae* revealed the central role of the ZIP (Zrt-, Irt-like protein) family of zinc transporters [30,31]. Selenites in yeast were found to be absorbed in a metabolism-dependent way, using a transporter of phosphate or monocarboxylate [32–34]. Gharieb and Gadd [32] also reported a fast, metabolism-independent process during selenite uptake in *S. cerevisiae*. Carrier-mediated passive transport might be crucial to selenite absorption by *Flammulina velutipes* [35].

In our previous studies, we found that the introduction of selenites into the medium strongly affected heavy metal uptake in mycelia. Interestingly, the effect was variable for different ions. For Ni(II) and Fe(III) ions, the metal content in harvested mycelia rose in proportion to the selenite concentration in the culture medium. However, the Zn content in harvested mycelia decreased when the concentration of selenites in the medium rose [24,36]. Importantly, in our experiments, the molarities of Zn(II) and  $\text{SeO}_3^{2-}$  in medium were not high enough to precipitate  $\text{ZnSeO}_3$  into the medium [37]. Most likely the inhibition of zinc accumulation by selenites (and vice versa) resulted from the formation of soluble Zn(II)-selenite complexes in the culture medium. The phenomenon of

Zn(II)-selenite complex compounds formation of different solubility, strength and charge, depending on the molar ratio of ions, has been described by Banks, Moriya and Sekine, Somer and Yilmaz and Feroci et al. [38–41]. Thus, determining the molar proportion of selenites and zinc (II) for the transport to the cell of both micronutrients has become an important task.

Considering all the factors described above, to obtain the new immunomodulatory preparation containing mushroom-derived polysaccharides, selenium and zinc we designed a multi-step approach:

- in the first step, we have optimized the composition of the culture medium to obtain the *L. edodes* mycelia enriched in Se and Zn in the assumed concentrations,
- then we have prepared mycelial extracts with defined polysaccharide, selenium and zinc content to test the expected biological activity,
- in the last stage of the work, we determined the effects of *L. edodes* mycelium extracts with different proportions of selenium and zinc concentrations on human T cells activation.

2. Materials and Methods

2.1. Microorganism and cultivation media

The *Lentinula edodes* (Berk.) Pegler strain used in this study was ATCC 48085. The seed culture was grown under conditions described in our previous works [7].

2.1.1. Supplementation of media and growth conditions in mycelial shake-flask cultures

The culture media contained glucose at 5%, yeast extract at 1%, soybean extract at 1.5%, and  $\text{KH}_2\text{PO}_4$  at 0.1% (w/v). The pH of the medium was 6.5. Mineral precursors (sodium selenite and/or zinc bromide) were added to the medium, as shown in Table 1.

**Table 1.** The protocol of supplementation of culture media in the series I-IV.

Series	Precursor	Concentration of precursor [mM]				
I	$\text{SeO}_3^{2-}$	0.1	0.2	0.4	0.6	0.8
	$\text{Zn}^{2+}$	0	0	0	0	0
II	$\text{SeO}_3^{2-}$	0.1	0.2	0.4	0.6	0.8
	$\text{Zn}^{2+}$	0.2	0.2	0.2	0.2	0.2
III	$\text{Zn}^{2+}$	0.1	0.2	0.4	0.6	0.8
	$\text{SeO}_3^{2-}$	0	0	0	0	0
IV	$\text{Zn}^{2+}$	0.1	0.2	0.4	0.6	0.8
	$\text{SeO}_3^{2-}$	0.2	0.2	0.2	0.2	0.2

As a reference, we also performed a series of studies in which the cultivation medium was non-enriched. The zinc and selenium content in the unenriched medium were the control levels in these studies.

Mycelia were grown in shake-flask cultures in 500 mL flasks containing 200 mL of medium. The fermentation medium was inoculated with 5% (v/v) of the seed culture. Cultures were incubated at 26 °C in a rotary shaker (New Brunswick Scientific, Edison, NY) at 120 rev/min for 10 days. Mycelia were harvested by filtration, washed three times with redistilled water, and freeze-dried. All experiments were conducted for five times to ensure reproducibility.

2.1. Determination of selenium and zinc in *L. edodes* mycelium

The determinations were performed after microwave mineralization.

2.2.1. Microwave mineralization procedure

A microwave digestion procedure was performed using a closed-vessel microwave system (Magnum II, ERTEC). A total of 0.02 g of sample was placed in a Teflon container and digested with

3 mL of 65% HNO<sub>3</sub> (Suprapur quality, Merck, Darmstadt, Germany) in the microwave digestion system according to a four-stage program: 17-20 atm for 3 min (60% of the microwave power), 27-30 atm for 5 min (80 % of the microwave power), 42-45 atm for 8 min (100% of the microwave power), and cooling for 10 min. When cool, the sample was diluted to 25 mL with deionized water. If necessary, the solutions were diluted several times. A blank digest was prepared in parallel.

#### 2.2.2. Determination of zinc content by high-performance ion chromatography (HPIC)

Chromatographic analyses were performed as described previously (6) on a Dionex DX-500 ion chromatograph (Dionex, Sunnyvale, CA, USA) equipped with an IP 25 isocratic pump, an IonPac CG 5A guard column, an IonPac CS 5A analytical column (250 x 4.6 mm I.D., 9 µm bead diameter, ethylvinylbenzene functionalized with both quaternary ammonium and sulfonate functional groups), a 25 µL injection loop, and a Dionex AD20 absorbance detector with a Pneumatic Controller (PC 10) post-column reactor. All samples were injected at least in triplicate. All measurements were made at 30 ± 1 °C. The eluent solution was 80 mM oxalic acid/100 mM tetramethylammonium hydroxide/50 mM potassium hydroxide at pH 4.7. The eluent flow rate was set to 0.3 mL/min. A solution of 4-(2-pyridylazo)resorcinol (PAR) was used as the postcolumn reagent and its flow rate was set to 0.15 mL/min. The Dionex PeakNet chromatography workstation was used for instrument control and data acquisition.

#### 2.2.3. Determination of the selenium content by reverse-phase high performance liquid chromatography (RPHPLC)

The reverse-phase high performance liquid chromatography method used for the determination of selenium was performed as described previously [41]. Namely, a 0.1% solution of diamionaphthalene (DAN) in 0.1 M HCl was prepared and stored in the dark at 4°C. The pH of each of digested sample dissolved in 8 mL of Suprapur water was adjusted to 1.8-2 by adding HCl or 7 M NH<sub>3</sub>·H<sub>2</sub>O, as needed. One milliliter of DAN was added to each sample and the mixtures were heated for 45 min at 75 °C. After cooling, 3 mL of cyclohexane was added, and the samples were shaken vigorously for 1 min to extract the fluorescent piasezenol. Prior to RP HPLC determination, the samples were stored in the dark. The sample solutions were diluted with cyclohexane as needed. A blank digest was manipulated similarly for use as a control. Selenium standard solutions (6.25-1000 ng Se/mL) were prepared under the same conditions. The fluorescence was a linear function of the concentration of Se in the tested range (correlation coefficient, R= 0.999). The RP HPLC conditions were as follows: eluent, acetonitrile: flow rate, 1.4 mL/min; temperature, 25 °C; injection volume, 20 µL. For fluorometric analysis, the excitation wavelength was 378 nm and emission wavelength were 557 nm. The piasezenol retention time was 3.1 min.

#### 2.3. Determination of the selenium and zinc content in mycelial water extracts by inductively coupled plasma mass spectrometry (ICP-MS)

The solvents and reagents used for the test had the highest purity class available on the market. Ultrapure water (18 MΩ cm<sup>-1</sup> resistivity) was taken from the Barnstead NANOPURE DIAMON UV System and used to prepare all standards and sample solutions. Suprapur 65% HNO<sub>3</sub> grade was used to dissolve the samples. A 10 µg/mL concentration multi-element solution was purchased from INORGANIC Ventures (Christiansburg, USA). The purity of the plasma gas (argon) and cell gas (helium) was greater than 99.999%.

Each sample was placed directly in the vessels sealed in the microdevice. 3 mL of HNO<sub>3</sub> was added. The mineralization of the samples was carried out in a high-pressure laboratory microwave oven (Milestone UltraWAVE T640). The heating program was carried out in two steps: In the first step, the temperature was increased linearly from 25 to 210 °C in 15 minutes. In the second stage, the temperature was kept at 210 °C for 8 minutes. After mineralization, the samples were diluted with water to a final volume of 100 mL. The Zn (Se) sample was further diluted (5 mL -> 50 mL)



The quadrupole ICP-MS 7800 (Agilent Technologies, Japan) equipped with an eight-field collision chamber was used for element analysis. The internal standard (In) has been added to compensate for any effects of acids or instrument drift. Measurements were made with a nickel probe and skimmer cones. The ICP-MS operating conditions are summarized in Table 2.

**Table 2.** Instrumental conditions of ICP-MS.

Parameter	Value
RF Power	1550 W
Plasma argon flow rate	15 L min <sup>-1</sup>
Auxiliary argon flow rate	0.9 L min <sup>-1</sup>
Nebulizer argon flow rate	1.03 L min <sup>-1</sup>
Cell gas flow rate	4.3 mL min <sup>-1</sup>
Dwell time	0.3 s
Sweeps	100
Number of readings per replicate	3
Conditions	Oxide 156/140 and doubly charged 70/140 < 2%

2.4. Preparation of the mycelial extracts

Hot water extracts were prepared from freeze-dried mycelium in a Soxhlet extractor. In order to investigate the effect of the micronutrient concentration ratio (zinc and selenium) on the immunomodulatory activity of the preparations, the following mycelial samples were selected for extraction:

- mycelium cultured in medium not enriched with micronutrients (sample 0),
- mycelium cultured in medium enriched only with selenium at a concentration of 0.8 mM (sample Se) ,
- mycelium cultured in medium enriched only with zinc at a concentration of 0.8 mM (sample Zn),
- mycelium cultured in medium enriched with selenium in a concentration of 0.8 mM and zinc in a concentration of 0.2 mM (sample Se/Zn),
- mycelium enriched with selenium in a concentration of 0.2 mM and zinc in a concentration of 0.8 mM (sample Zn/Se).

After 8 hours of the extraction of 0.5 g of mycelium with 20 mL of water, the extract was evaporated under the reduced pressure. The freeze-dried extracts were used for tests of the chemical composition (polysaccharide, Zn and Se content) and redissolved in water to the appropriate concentration, for tests of biological activity.

2.5. Determination of the carbohydrate content in the mycelial extracts

The determination was performed by the phenol-sulphuric acid method (Dubois method) [42]. One milliliter of the sample (water extract at a concentration of 0.3 mg/mL) was mixed with 1 mL of 5% phenol solution and 5 mL of concentrated H<sub>2</sub>SO<sub>4</sub>. The mixture was kept at room temperature for 10 minutes, and then heated at 30 °C for 20 minutes. Absorbance at 490 nm was measured. A calibration curve was constructed with different concentrations of 1:1 w/w galactose-mannose mixture (25-100 µg/mL) as the standard.

2.6. Determination of the effects of mycelial extracts on T cells activation

2.6.1. PBMCs isolation, culture and stimulation

Blood samples (8 mL) were collected from seven healthy donors. All blood samples were commercially obtained from the Regional Blood Centre in Warsaw. Isolation of peripheral blood mononuclear cells (PBMCs) was performed by density gradient centrifugation on Histopaque-1077 (Sigma-Aldrich, St. Louis, MO, USA). PBMCs were collected at the interface of plasma and

Histopaque, and washed twice with phosphate buffered saline (PBS, Sigma-Aldrich, St. Louis, MO, USA). The separated PBMCs were resuspended in RPMI 1640 medium (Gibco, Gaithersburg, MD, USA) containing antibiotic-antimycotic solution (1.5% penicillin-streptomycin-amphotericin, Invitrogen, Waltham, Massachusetts, USA) and 10% human serum (Gibco, Gaithersburg, MD, USA) and counted. PBMCs ( $2 \times 10^5$  cells/well) were cultured in 96-well flat-bottom microplates (Nunc, Paris, France) as described in detail in our previous paper [43]. Briefly, cells were stimulated with anti-CD3 Ab (coated on plate wells, 0.75  $\mu\text{g/mL}$ , BD Pharmingen, USA) or Dynabeads™ Human T-Activator CD3/CD28 (2  $\mu\text{L}$  per well, ratio 2:5, Gibco, MA, USA) and incubated with *L. edodes* mycelium extracts at concentration of 100  $\mu\text{g/mL}$ . PBMCs were cultured for 24 h at 37 °C and 5% CO<sub>2</sub> in a humidified incubator.

### 2.6.2. Flow cytometry

For flow cytometry  $2 \times 10^5$  cells from each well were washed once in PBS and stained with antibodies against specific surface antigens (all from BD Biosciences, San Jose, CA, USA): CD3-PerCP (SK7 clone), CD4-APC-Cy7 (SK3 clone), CD8-APC (SK1 clone), CD69- BV711 (FN50 clone, Horizon™), and CD25- FITC (2A3 clone). Prior to experiments each antibody was titrated in order to achieve highest signal to background ratio. Samples were incubated in the dark for 15 min in room temperature. Next, cells were rinsed in 1 mL of PBS without Ca<sup>2+</sup> and Mg<sup>2+</sup> with 0.01% sodium azide and resuspended in 60  $\mu\text{L}$  PBS without Ca<sup>2+</sup> and Mg<sup>2+</sup> with 0.01% sodium azide. Cut off values for each positive population were based on fluorescence minus one experiment.

Relative frequencies of CD69 and CD25 expression were assessed on singlets populations of CD3+CD4+ T cells and CD3+CD8+ T cells. Flow cytometry was performed using a DxFLEX Flow Cytometer (Beckman Coulter, Brea, CA, USA) and analyzed with CytExpert software.

### 2.7. Statistical analysis

Statistical analyses (biotechnological and analytical part) were performed using StatSoft, Inc. (2014) STATISTICA (data analysis software system), version 12. All computations were applied at a significance level of 0.05.

All data in groups were first tested for normality using the Shapiro-Wilk test. To compare the means of two groups, a t-test was applied if the variances of the two populations were equal, otherwise a Cochran-Cox test was applied. To check the homogeneity of variance, the Brown-Forsythe test was used.

Statistically significant differences between more than two groups were assessed using one-way analysis of variance preceded by checking the assumptions of normality and homogeneity of variance. For multiple comparisons, the Tukey post-hoc test was performed, while for comparing the control group to each of the others, Dunnett's test was used.

Flow cytometry data was acquired and interpreted using CytExpert software (Beckman Coulter). Acquired data was then analyzed and visualized with GraphPad Prism 9.5.0 software. To determine normality of data sets Shapiro-Wilk and Kolmogorov-Smirnov tests with  $\alpha = 0.05$ , and each set was considered to have normal distribution when both tests had  $p > 0.05$ . To compare data sets repeated measures ANOVA test with Geisser-Greenhouse correction was made for data sets with normal distribution and Friedman tests for those without normal distributions, both with  $\alpha = 0.05$ . Results were considered significantly important with  $p < 0.05$ .

## 3. Results

### 3.1. Accumulation of Se and Zn in mycelium of *L. edodes*

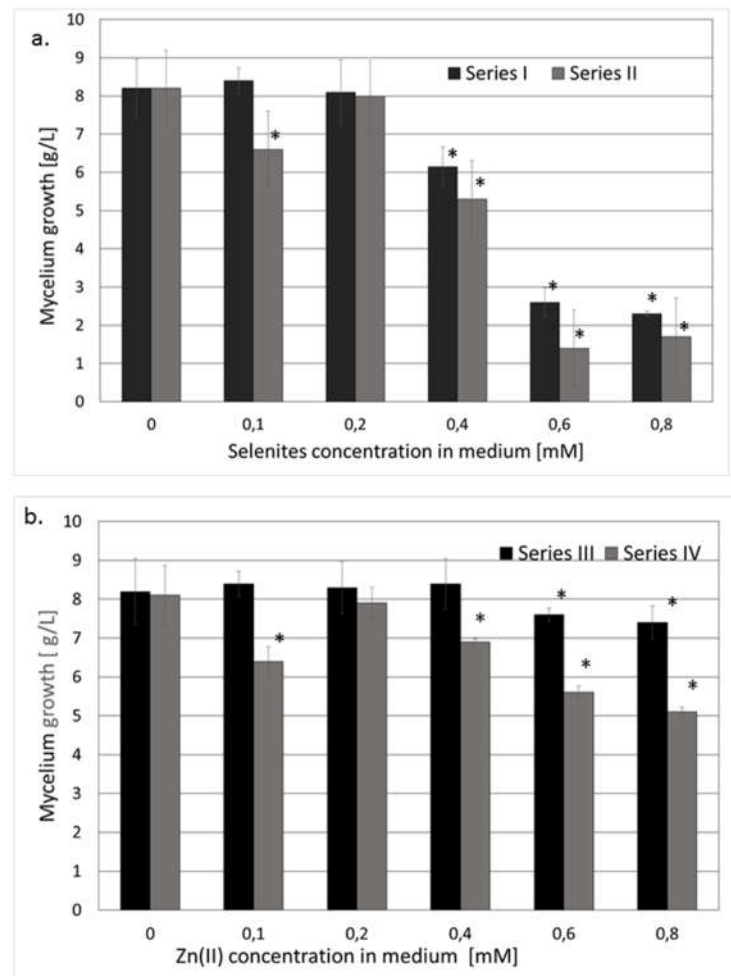
#### 3.1.1. Reference series and mycelial growth

The cultivation of the mushroom mycelium in medium non-enriched in Na<sub>2</sub>SeO<sub>3</sub> and/or ZnBr<sub>2</sub> afforded 7-8 g of dry weight of mycelium per liter of culture medium after 10 days. The selenium and zinc concentrations in the medium unenriched in sodium selenite and zinc bromide medium were

1.138  $\mu\text{M}$  and 0.036 mM, respectively. The determination was performed after mineralization of a medium sample with concentrated nitric acid. The total Se and Zn contents were determined without speciation.

The selenium and zinc contents in the reference series of dried mycelium (mycelium cultivated in unenriched medium) equaled 1.27 and 289  $\mu\text{g/g}$ , respectively. The mycelial growth in enriched media was only weakly affected by increasing the concentrations of the supplements.

A depressive effect was observed only in cultures enriched with sodium selenite or zinc bromide in concentrations higher than 0.4 mM. The mycelium growth significantly decreased when the second ion (selenite or zinc) was added to the medium (Figure 1a,b), except for the cultures with a zinc(II)/selenite molar ratio of 1:1.

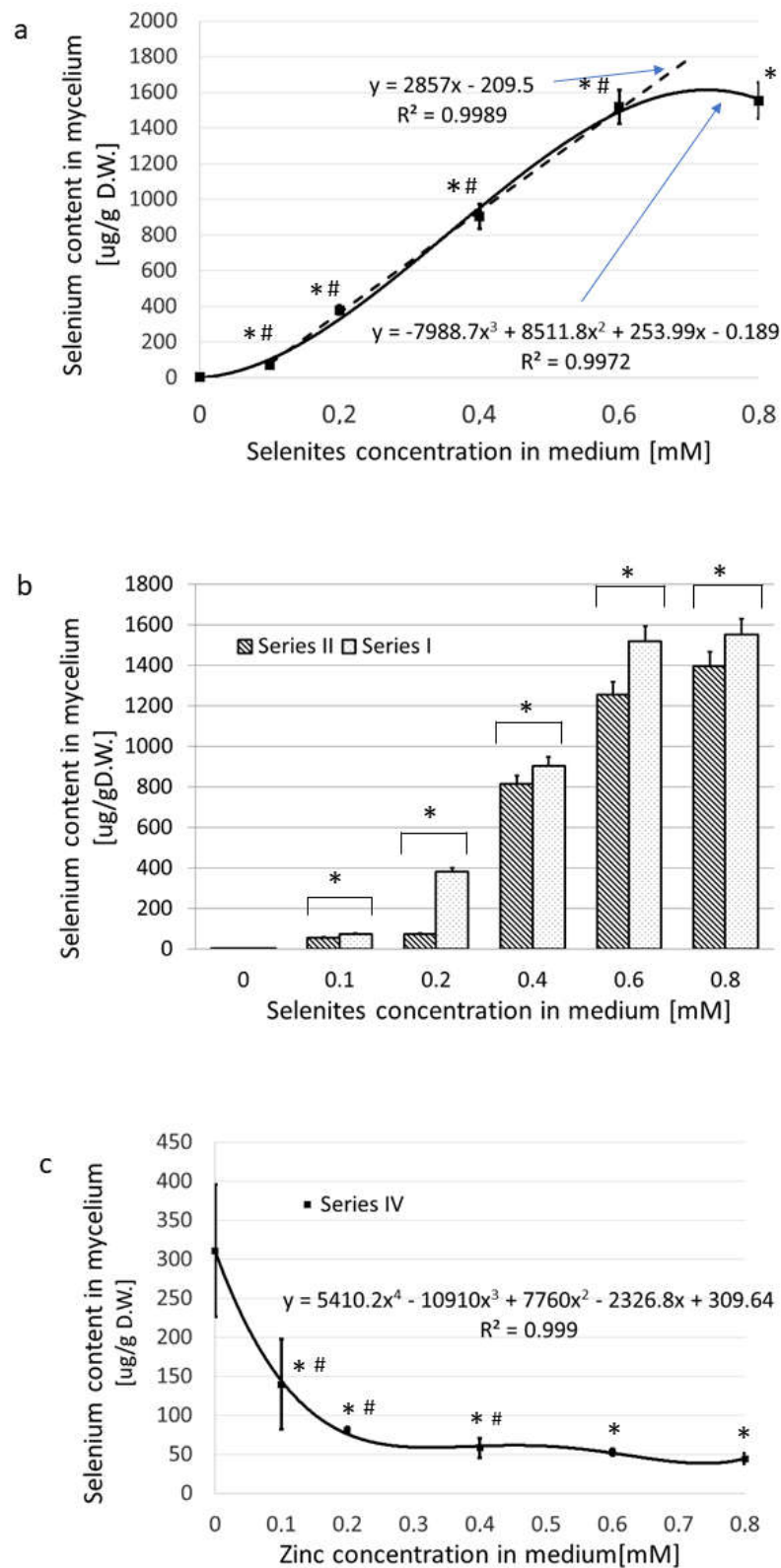


**Figure 1.** The mycelial growth of *L. edodes*. a. In media enriched in selenites exclusively (Series I) versus in medium also containing Zn(II) (Series II). b. In media enriched in Zn(II) exclusively (Series III) versus in medium also containing selenites (Series IV). (\*) values significantly different from the reference culture cultivated in non-enriched medium ( $p < 0.05$ ).

### 3.1.2. The accumulation of Se exclusively versus in the presence of Zn(II)

In the Series I (media supplemented with  $\text{Na}_2\text{SeO}_3$ ), the uptake of selenium to the submerged cultivated mycelium was very effective, as it was found in our previous works [45,46]. Addition of 0.8 mmol/L of sodium selenite to the medium (the maximum concentration used) yielded 1554  $\mu\text{g}$  of selenium per 1 g of mycelial dry weight (Figure 2a, Table S1).





**Figure 2.** Accumulation of selenium into the mycelium of *L. edodes* from media enriched in selenites exclusively versus in medium also containing Zn(II). a. The content of selenium absorbed by the mycelium as a function of the selenite concentration in medium enriched exclusively with sodium selenite. (\*) values significantly different from the reference culture cultivated in non-enriched

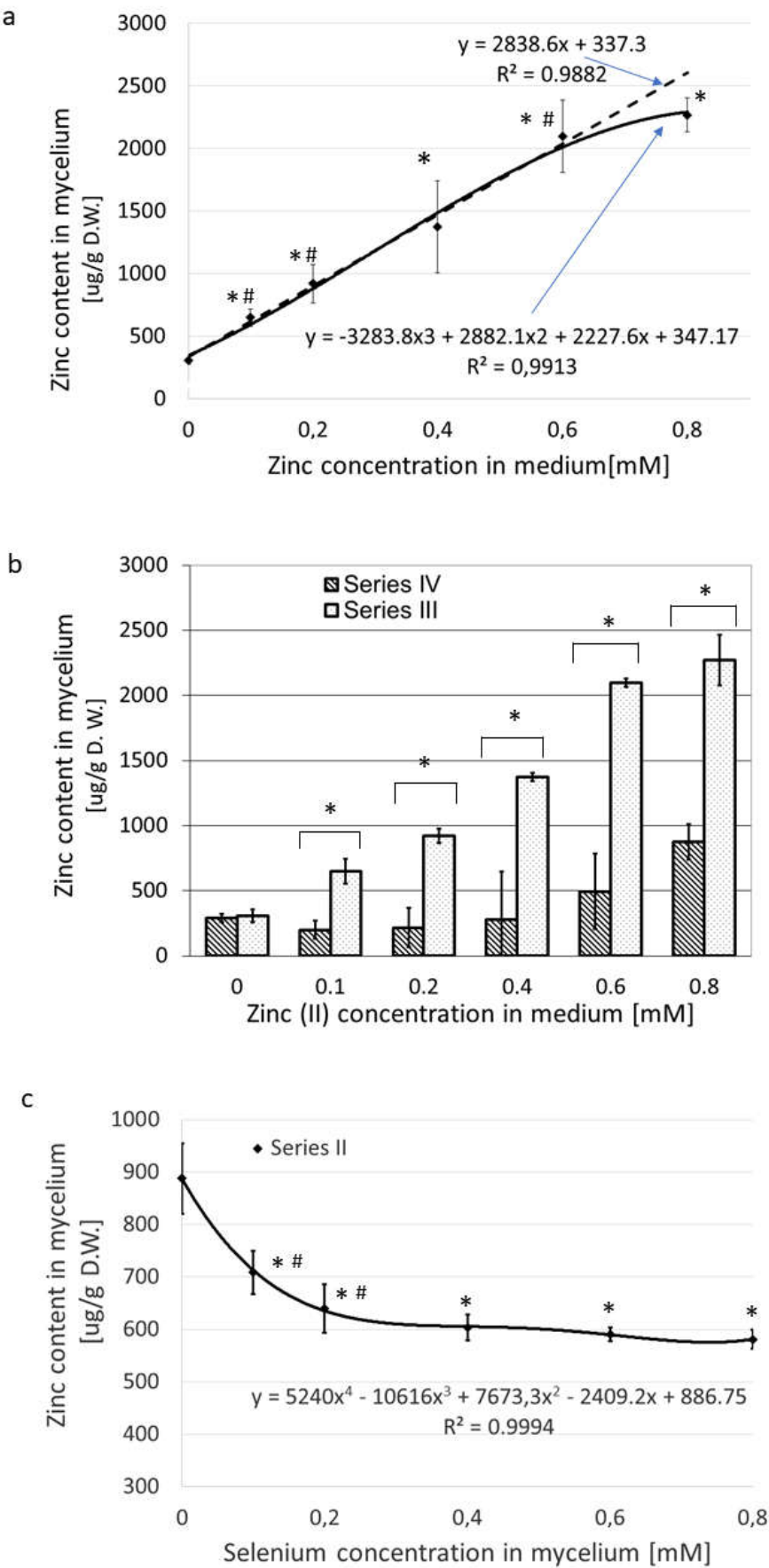
medium ( $p < 0.05$ ). (#) values significantly different versus the previous culture supplemented with a lower concentration of selenite ( $p < 0.05$ ). b. Comparison of Se accumulation from medium enriched exclusively with selenites (Series I) versus in the presence of 0.2 mM Zn(II) (Series II). (\*) values of Series I significantly different from Series II ( $p < 0.05$ ). c. Impact of increasing Zn(II) concentration in the medium (0-0.8 mM) on the accumulation of selenium from medium enriched in 0.2 mM sodium selenite (Series IV). (\*) values significantly different from the reference culture cultivated in non-enriched medium ( $p < 0.05$ ). (#) values significantly different versus the previous culture supplemented with a lower concentration of selenite ( $p < 0.05$ ). In each panel, the data are presented as the mean  $\pm$  S.D. of five observations.

The content of selenium in the mycelial dry weight correlated well with the  $\text{Na}_2\text{SeO}_3$  concentration in the medium for concentrations ranging from 0.1-0.6 mM. At levels higher than 0.6 mM, no additional uptake of Se from the medium occurred (Figure 2a).

In Series II (medium enriched in  $\text{Na}_2\text{SeO}_3$  and containing zinc bromide at 0.2 mM), equimolar concentrations of zinc and selenites in the medium resulted in an 83% decrease ( $p < 0.05$ ) in the selenium content in mycelium as compared with Series I (Figure 2b). In the presence of an excess of selenites, the selenium absorption was almost as effective as in Series I (decrease by about 10%,  $p < 0.05$ ) (Figure 2b). Selenium absorption decreased when the medium contained a constant amount of selenite (0.2 mM) and that the concentration of zinc rose from zero to 0.8 mM (Series IV) (Figure 2c). The values of selenium absorption dropped from 311 to 45, (86%,  $p < 0.05$ ). The greatest decrease in the concentration of selenium in the mycelium (by 74%) was observed when the zinc concentrations in the culture medium were 0.1-0.2 mM, thus providing a selenite/zinc(II) molar ratios of 2:1 and 1:1, respectively. Further increases of the concentration of zinc up to 0.8 mM (affording a 4:1 ratio of selenite/zinc(II)) did not further reduce the selenium content (Figure 2c).

### 3.1.3. Zn(II) accumulation exclusively versus in the presence of $\text{SeO}_3^{2-}$

Figure 3a and Table S2 show the dynamics of zinc absorption when Zn alone was added to the medium in increasing concentrations (Series III). When the concentration of  $\text{ZnBr}_2$  in the medium increased from nearly zero to 0.8 mM, the values of absorbed zinc rose from 308 to 2370  $\mu\text{g/g}$  dry weight. The content of zinc in the mycelium correlated well with its concentration in medium in the concentration range 0.1-0.6 mM (Figure 3a).



**Figure 3.** Accumulation of zinc in the mycelium of *L. edodes* from media enriched in Zn(II) exclusively versus in the presence of selenites (0-0.8 mM Se). a. The effect of increasing the zinc content in the medium on the amount of Zn accumulated in the mycelium. The culture medium contained only zinc bromide and no added selenium. (\*) values significantly different from the reference culture cultivated in non-enriched medium ( $p < 0.05$ ). (#) values significantly different versus the previous culture supplemented with a lower concentration of Zn(II) ( $p < 0.05$ ). b. Comparison of Zn accumulation into the mycelium when the medium was enriched exclusively in Zn(II) (Series III) versus when 0.2 mM sodium selenite was also present in the medium (Series IV). (\*) values of Series III significantly different from Series IV ( $p < 0.05$ ). c. Impact of increasing selenite concentration in the medium on zinc accumulation from medium enriched in 0.2 mM zinc bromide (Series II). (\*) values significantly different from the reference culture cultivated in non-enriched medium ( $p < 0.05$ ). (#) values significantly different versus the previous culture supplemented with a lower concentration of Zn(II) ( $p < 0.05$ ). The data are presented as the mean  $\pm$  S.D. of five observations.

The concentrations higher than 0.6 mM zinc bromide hampered Zn uptake from the medium. This effect was like that recorded for selenium uptake and suggests feedback inhibition at excessive concentrations, although another mechanism, such as saturation, may also be possible. When  $\text{Na}_2\text{SeO}_3$  was held at a constant concentration of 0.2 mM in the medium and the  $\text{ZnBr}_2$  concentration was increased over the range from nearly zero to 0.8 mM (Series IV), we noted an initial drop in the zinc absorption into the mycelium, but the change was not significant (Figure 3b, series IV.) Under these conditions, as the Zn content of the medium increased, the absorbed level of Zn also increased by nearly three-fold in the medium supplemented with 0.8 mM  $\text{ZnBr}_2$ .

When the medium contained a fixed amount of zinc(II) (0.2 mM) and the concentration of selenium was varied from 0 to 0.8 mM (Series II), the values of absorbed zinc dropped from an initial high of 888 to as low as 591, approximately 25% ( $p < 0.05$ ) (Figure 3c). Under these experimental conditions, the greatest decrease in the concentration of zinc in the mycelium was observed with the lower selenium levels, 0.1 and 0.2 mM of selenites, in the culture medium, or with zinc(II)/selenite molar ratios of 2:1 and 1:1. Further increases in the concentration of selenium of up to 0.8 mM (four-fold excess relative to the concentration of zinc(II)) did not reduce the absorbed zinc content significantly (Figure 3c).

### 3.2. Composition of the water extracts

Table 3. presents the data on the concentration of selenium, zinc and saccharides in the *L. edodes* mycelial hot water extracts. The results are given in comparison with the Zn and Se concentration in the extracted mycelia.

**Table 3.** The composition of the extracts subjected to biological activity tests.

Sample acronym	Molar ratio of medium supplements (mM)		Concentration of Se and Zn in mycelium (ug/g)		Concentration of Se, Zn and saccharides in mycelial water extracts (mg/g)		
	$\text{SeO}_3^{2-}$	Zn (II)	Se	Zn	Se	Zn	Saccharides
0	0	0	2.52 (0.17)	298.10 (29.02)	0.39 (0.06)	92.65 (0.91)	196.40 (6.91)
Se	0.8	0	1553.91 (102.1)	181.92 (19.46)	203.60 (5.18)	134.81 (4.42)	139.64 (4.31)
Se/Zn	0.8	0.2	1395.89 (16.86)	591.25 (18.41)	199.39 (7.99)	152.04 (6.07)	146.60 (2.90)
Zn/Se	0.2	0.8	44.59 (6.72)	874.94 (194.16)	26.67 (0.56)	212.42 (4.42)	142.16 (9.72)

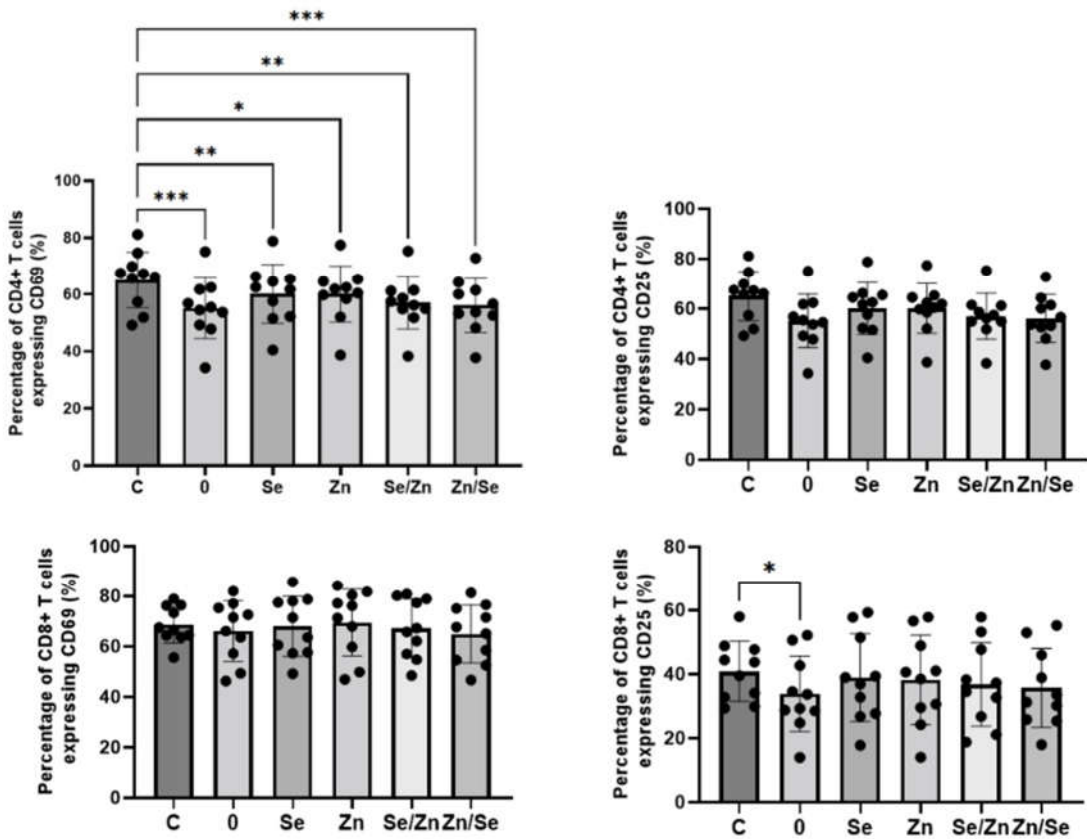
Zn	0	0.8	3.01 (0.65)	2370.32 (235.49)	7.07 (0.09)	393.15 (4.46)	188.53 (4.64)
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Standard deviations are given in parentheses.

3.3. Immunological activity of mycelial extracts

Effective immune response to infection depends on the activation of immunocompetent cells [47]. Activated T lymphocytes express on their surface numerous molecules, including CD69, and CD25 (also known as interleukin-2R $\alpha$ ) [48]. CD69 is the earliest antigen upregulated four hours after activation. It is essential for T cell proliferation and survival. CD25 is upregulated 12–24 h after activation and initiates cells proliferation and differentiation [49–51].

The effects of mycelial extracts on the expression of activation markers on CD4+ and CD8+ T cells stimulated with anti-CD3 Ab is presented in Figure 4. All analyzed extracts significantly downregulated the percentage of CD4+CD69+ T cells, however, extract not enriched with micronutrients (sample 0) showed the strongest effect in comparison to control (C: 65.07  $\pm$  9.83%; sample 0: 55.11  $\pm$  10.68%,  $p$  = 0.0004; sample Se: 60.11  $\pm$  10.39%,  $p$  = 0.0049; sample Zn: 60.10  $\pm$  9.90,  $p$  = 0.0358; sample Se/Zn: 57.00  $\pm$  9.19%,  $p$  = 0.0028; sample Zn/Se: 56.07  $\pm$  9.53%,  $p$  = 0.0008). The statistically significant impact on CD25 expression on CD4+ T cells was not observed ( $p$  > 0.05), however, this marker tended to be downregulated by the non-enriched extract. All analyzed extracts did not affect the expression of CD69 marker on CD8+ T cells ( $p$  > 0.05), but non-enriched fraction significantly downregulated the percentage of CD8+CD25+ T cells (40.84  $\pm$  9.36% vs. 33.90  $\pm$  11.78%,  $p$  = 0.0406).

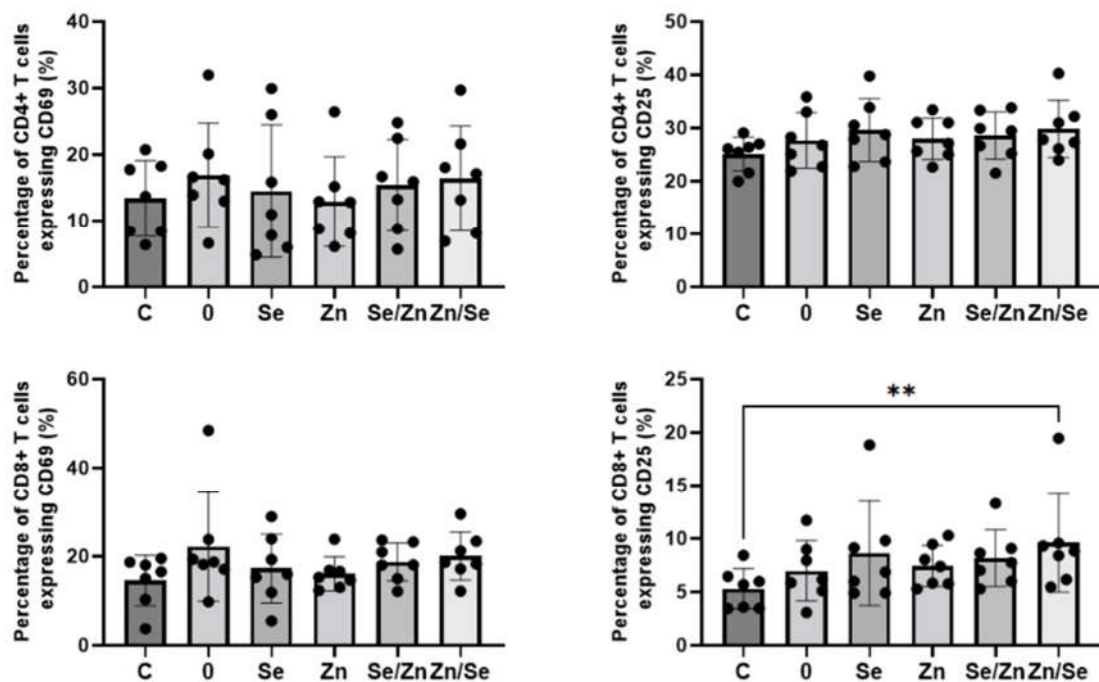


**Figure 4.** Effects of *L. edodes* mycelium water extracts with different proportions of selenium and zinc concentrations on the activation of human CD4+ (top row) and CD8+ (bottom row) T cells stimulated with anti-CD3 Ab. PBMCs from seven donors were stimulated and treated with extracts in the



concentration of 100  $\mu\text{g/mL}$  for 24 h. The expression of CD69 and CD25 markers on the surface of T cells was detected by flow cytometry. Statistical differences were considered when  $p < 0.05$ . \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ .

The effects of *L. edodes* mycelial extracts on the expression of activation markers on CD4+ and CD8+ T cells stimulated with anti-CD3/CD28 Abs is presented in Figure 5. When cells were stimulated with a dual signal, the number of CD4+ T cells expressing CD69, and CD25 marker was higher, however, the observed changes were not statistically significant ( $p > 0.05$ ). All analyzed extracts showed no effects on the expression of CD69 marker o CD8+ T cells ( $p > 0.05$ ), but extract enriched with Zn and Se (sample Zn/Se) significantly increased the number of CD8+CD25+ T cells ( $5.28 \pm 1.89\%$  vs.  $9.60 \pm 4.63\%$ ,  $p = 0.0017$ ).



**Figure 5.** Effects of *L. edodes* mycelium water extracts with different proportions of selenium and zinc concentrations on the activation of human CD4+ (top row) and CD8+ (bottom row) T cells stimulated with anti-CD3/CD28 Abs. PBMCs from seven donors were stimulated and treated with extracts in the concentration of 100  $\mu\text{g/mL}$  for 24 h. The expression of CD69, and CD25 markers on the surface of T cells was detected by flow cytometry. Statistical differences were considered when  $p < 0.05$ . \*  $p < 0.05$ ; \*\*  $p < 0.01$ .

#### 4. Discussion

As stated in the Introduction, the first objective of our study was to optimize the conditions for simultaneous accumulation of zinc and selenium in the mycelial cultures of *L. edodes*, to obtain an immune-active preparation containing  $\beta$ -D-glucans and two micronutrients necessary for proper functioning of the immune system. The mutual blockade of the transport of selenium and zinc to animal and plant cells is a well-known phenomenon, although its mechanism is not entirely clear [52]. Thus, we planned to check whether such a phenomenon, which could hinder the achievement of the assumed goal (obtaining a mycelium enriched in both, organic selenium, and zinc compounds) occurs in submerged cultured fungal cells.

As for the mutual influence of selenites and zinc(II) ions on the accumulation of Se and Zn in *L. edodes* mycelial cultures: cumulation of selenium in the presence of zinc, and of zinc in the presence

of selenium strongly decreased in comparison to amount the individual ion absorbed when the medium was supplemented with that ion alone (Fig.2. and Fig 3.). The mutual inhibitory effect of zinc ions and selenites on transport to the fungal cell is therefore like the effects described for other organisms [52]. It turned out to be confusing, however, that the strongest indication comes from the results obtained at the  $\text{Zn}^{2+}$  to  $\text{SeO}_3^{2-}$  concentration ratio of 1:1 (0.2mM:0.2mM). Further, the substantial excess of the second ion did not cause a proportional inhibition of the accumulation process (Fig.2, Fig.3.). Since the molar ratio of ions in the culture medium influenced the accumulation of selenium and zinc in fungal cells more significantly than the absolute values of ion concentrations, we hypothesized that interactions between ions may be responsible for this phenomenon. We assumed that formation of the zinc-selenite complexes of different charges would very likely inhibit the first stage of the accumulation process, i.e., biosorption. This hypothesis was confirmed in our further research, however, due to the extensiveness of the material, it will be described in a separate publication.

Our previous studies have shown that the optimum concentration of sodium selenite in the cultivation medium, providing the *L. edodes* mycelium enriched in organically bounded selenium was 0.12 - 0.25 mM (10-20  $\mu\text{g}$  of Se/L) [44,45]. To provide the same selenium content in the mycelium when zinc (II) ions are present in the medium, the culture needs to be enriched in substantially higher amount of sodium selenite (above 0.4 mM). At this concentration of selenites, the strength of zinc ions should be lower than 0.4 mM to avoid the zinc selenite precipitation (the ion product would not exceed the solubility product of zinc selenite). Taking into consideration the strong inhibitory effect of equimolar concentrations of selenites and zinc(II) on accumulation of both elements, the zinc concentration of 0.2-0.3 mM should be considered optimum. However, the amount of Zn in the mycelium cultured under these conditions will be much lower than in cultures supplemented with zinc salts exclusively.

Further experiments were aimed at examining whether the enrichment of the *L. edodes* preparations in selenium and zinc would give a stronger immunomodulatory effect than in each of these elements separately. Thus, for the preparation of the hot water extracts subjected to the immunological experiments, were selected mycelia enriched exclusively in selenium, exclusively in zinc and in both elements in two different proportions (Table 3). The choice of the  $\text{Zn(II)}$  and  $\text{SeO}_3^{2-}$  concentration ratio in the culture medium (0.8:0, 0.8:0.2, 0.2:0.8, 0:0.8 mM) resulted from the data presented above (subsection 3.1.3.).

The results of the ICP-MS analysis showed that the concentrations of Se and Zn in the extracts were almost linearly dependent on their concentrations in the mycelium (Table 3, Figure 5). However, they were almost 1000 times higher in the extracts than in mycelium (mg/g versus  $\mu\text{g/g}$ ). To sum up: hot-water extractability of the derivatives of selenium and zinc from the *L. edodes* mycelium was effective. Nevertheless, at high concentrations of Se and Zn in the mycelium the extractability of these elements clearly decreased. It's probably a consequence of binding to the structure of non-extractable proteins and polysaccharides.

The concentration of polysaccharides in the aqueous extracts clearly depended on the concentration of zinc and selenium in the mycelium. The highest concentration was in the extracts from the mycelium not supplemented with Se and/or Zn. Zinc supplementation had a much weaker effect on the concentration of polysaccharides in extracts than selenium supplementation. The lowest concentration of polysaccharides was found in extracts from mycelium supplemented with selenium alone. In our previous studies, we found that supplementation of cultures with high concentrations of selenium changed the structure of the fungal cell wall, significantly reducing the content of extractable polysaccharides. The current results confirm these findings.

The results obtained in immunological tests suggest that the highest biological activity possess *L. edodes* water extracts not supplemented with Se or Zn (sample 0) or those where mycelium was enriched with Se concentration of 0.2 mM and Zn in a concentration of 0.8 mM (sample Zn/Se). Both analyzed T cells populations are essential in the protective immunity against viral infections, including SARS-CoV-2 [53,54] In our previous study we demonstrated that the direction of *L. edodes* polysaccharides biological activity depends on the type of cells stimulation [43]. The present study

confirms these observations. When cells were stimulated only with anti-CD3 Ab, extracts decreased T cells activation. In contrast, when cells were stimulated with a dual signal (anti-CD3/CD28 Abs), which is more physiological [55], extracts enhanced their activation. In conclusion, there is insufficient amount of evidence to confirm that Se or Zn supplementation to mycelial cultures of *L. edodes* enhances the immunomodulatory properties of water extracts. Therefore, future studies on larger sample focusing on identification of intracellular signaling pathways are needed.

## 5. Conclusions

We have successfully optimized the composition of the culture medium to obtain the *L. edodes* mycelia enriched in Se and Zn in the assumed concentrations. The molar ratio of ions in the culture medium influenced the accumulation of selenium and zinc in fungal cells more significantly than the absolute values of ion concentrations. The obtained results indicate that interactions between selenate and zinc ions in the culture medium (complexation) influence the process of accumulation. This has an impact on the final concentration of microelements in biomass and in the designed dietary supplements.

In the mycelial preparations (water extracts) tested in the present study, the content of selenium and zinc was proportional to the content in the mycelium, although a significant part of Zn and Se was present in the mycelium in a form not water - extractable form (probably bound to proteins).

The immunological activity direction of the mycelial extracts strongly depends on the type of cells stimulation. More importantly, the effect of the mycelial extracts containing polysaccharides as well as zinc and selenium on the activation of T lymphocytes indicates the key role of fungal polysaccharides: the strongest effect was shown by preparations containing the highest concentration of these compounds, regardless of the presence of selenium and zinc. In case of preparations enriched in selenium and zinc, a significant predominance of the concentration of zinc over the concentration of selenium seems to be more advantageous. The selenium and zinc content in the examined preparations modified the immunomodulatory activity of mycelial polysaccharides, however, the mechanisms of action of various active ingredients of the mycelial extracts seem to be different.

In our opinion, however, there is insufficient amount of evidence to confirm that Se or Zn supplementation to mycelial cultures of *L. edodes* significantly enhances the immunomodulatory properties of water extracts. Therefore, future studies on larger sample focusing on identification of intracellular signaling pathways are needed.

### Supplementary Materials:

**Author Contributions:** Conceptualization, M.Ka., J.T.; methodology, M.Ka., J.T. (biotechnology), R.Z. and B.K. (immunology), A.B. (HPIC analysis), P.D. (ICP-MS analysis); A.R. (T cells activation experiments); validation, A.R., M.K., B.K.; formal analysis, J.T.; investigation, M.Ka., E.D., A.B., M. Kr., S.G.-J., B.K., A.R., A.M.P., K.T., A.W.; resources, M.Ka.; data curation, M.Ka., E.M., B.K., A.R., M.Kl., A.B.; writing—original draft preparation, M.Ka., J.T., B.K.; writing—review and editing, M. Ka., J.T., R.Z., B.K., M.Kr.; visualization, J.T., A.R., P.P. supervision, J.T.; project administration, J.T.; funding acquisition, S.G.-J., J.T. All authors have read and agreed to the published version of the manuscript.

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**Data Availability Statement:** The data presented in this study are available on request from the corresponding author and co-authors.

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