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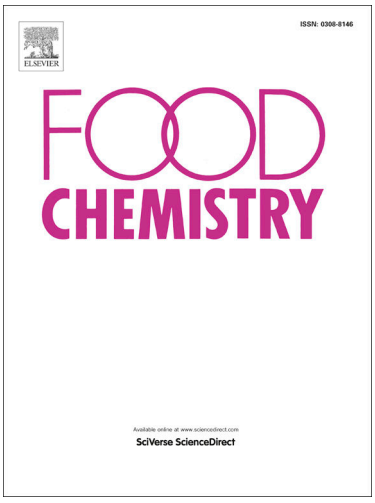


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# Fermentation characteristics of novel *Coriolus versicolor* and *Lentinus edodes* kombucha beverages and immunomodulatory potential of their polysaccharide extracts

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## Highlights

- Novel kombucha was prepared from *L. edodes* and *C. versicolor* medicinal mushrooms
- Polysaccharide extracts content of novel kombucha depend on mushroom species
- The polysaccharide extracts stimulated proliferation of human PBMC at non-toxic doses
- Polysaccharide extracts downregulated Th2 cytokines and IL-10
- These medicinal mushroom-based kombucha preparations are potential nutraceuticals

## Abstract

Medicinal mushrooms, *Coriolus versicolor* and *Lentinus edodes* are extremely attractive as nutraceuticals. Here we used fruiting bodies to prepare novel kombucha beverage. Microbiological,

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physicochemical and chemical properties were monitored for eleven days, while the immunological properties of kombucha polysaccharide extracts were determined in peripheral blood mononuclear cell (PBMC) cultures. FTIR analysis of polysaccharide extracts showed dominant presence of polysaccharides, in addition to phenols, lipids and proteins. *C. versicolor* kombucha extract displayed more complex polysaccharides, and a higher content of total polysaccharides, phenols and flavonoids compared to *L. edodes* kombucha extract. The extracts were not cytotoxic for PBMC *in vitro* up to 500 μg/ml, while immunomodulatory effects depended on their chemical compositions. The most prominent effect was on the reduction of Th2 cytokines and IL-10 in PBMC cultures. Based on these results, novel kombucha products could be recommended as functional beverages or nutraceuticals with potentially beneficial immunomodulatory effects in allergies.

**Keywords:** *Coriolus versicolor*; *Lentiunus edodes*; Medicinal mushrooms; Kombucha; Immunomodulation; Cytokines

## 1. Introduction

Food industry trends are changing as a result of increased consumers' awareness to meet a balance between active life-style and high nutritional demands for daily consumed food. Nowadays, it is expected from food products to express positive health effects on human organism, which influences rised interest to determine their role in disease prevention (Ghoshal & Kansal, 2019).

Mushrooms have been used in traditional Chinese medicine for balanced nutrition achievement, health improvement, as well as for prevention or treatment of diseases, for over 3000 years. So far, more than 130 therapeutic functions (antimicrobial, antioxidative, hepatoprotective, antidiabetic, hypotensive, etc.) were detected in various cultivated or wild medicinal mushroom species (Siu, Chen & Wu, 2014; Matijašević et al., 2016). Even various mushrooms and their biologically active compounds (*i.e.*

polysaccharides, polypeptides, proteins, phenols, flavonoids, sterols, etc.) have been proved to express pharmacological properties, their application in food systems is still scarce (Siu, Chen & Wu, 2014; Matijašević et al., 2016). Namely, a great number of researches related to the biological activities of non-edible medicinal mushrooms were focused on the extraction of biologically active compounds, while their possible inclusion in the process of food production is neglected (Lin, Lai, Yu, Chen, Chang, Lo, & Hsu, 2008; Kozarski et al., 2012; Duvnjak et al.,2016; Matijašević et al., 2016). As an example, commercial preparations (polysaccharopeptide krestin, PSK and polysaccharopeptide, PSP) from *Coriolus versicolor*, non-eadible medicinal mushroom, are available on the market (Cui & Chisti, 2003). Among others, it was noticed that these preparations exhibit immunomodulatory activities (Lin et al., 2008). Additionally, the study of Lee, Yang and Wan (2006) showed that this medicinal mushroom stimulates the production of certain interferons (*i.e.* IFN-γ), interleukins (*i.e.* IL-2, IL-1β) and tumour necrosis factor (*i.e.* TNF-α). On the other hand, *Lentinus edodes*, medicinal and edible mushroom was already used for the production of alcoholic beverage (Lin, Huang, Mau, Liou & Fang, 2010). Polysaccharide Lentinan, isolated from *L. edodes*, in combination with chemotherapy was shown to downregulate anti-inflammatory cytokines IL-4, IL-5 and IL-10 and upregulate pro-inflammatory cytokines such as IL-2, IL-6 and IL-12, in patients with esophageal carcinoma (Wang, Bi, Zou & Gu, 2012).

Kombucha is a fermented beverage, which has appeared around 220 BC in Manchuria, northeast Chinese region, wherefrom it was spread around the world (Velićanski, Cvetković, Markov, Šaponjac, Šaponjac & Vulić, 2014). Alcoholic and acetic acid fermentation, that intertwine during the kombucha fermentation, create specific organoleptic properties of obtained beverage. The final product is non-alcoholic, refreshing and sparkling beverage with pleasantly sour taste and aroma (Chen & Liu, 2000; Chakravorty, Bhattacharya, Chatziontas, Chakraborty, Bhattacharya & Gauchhui, 2016). Although kombucha is traditionally produced in substrates prepared from black or green tea, it was shown that it

can be successfully produced by using other nitrogen sources such as plants, *e.g. Echinacea purpurea* L., *Satureja montana* L., *Melissa officinalis* L., as well as milk, wine, soda, etc. (Velićanski et al., 2014; Sknepnek et al, 2018). Compared to many plants, mushrooms have a better protein quality, which refers to high amount of essential amino acids (Meenu & Xu, 2019). It was shown that *L. edodes* extract contains significant yeast assimilable nitrogen, which was sufficient for yeast cell proliferation and alcoholic beverage production with unique flavour and functionality (Lin et al., 2010). Our previous research confirmed the successful kombucha fermentation on *Ganoderma lucidum* medicinal mushroom substrate, after hot water extraction, significantly enhancing its antibacterial and antioxidant activity (Sknepnek et al., 2018).

Beverages are known as a great carrier and distributors of nutrients, bioactive components and other valuable components to consumers (Ghoshal & Kansal, 2019). Considering highly attractive chemical composition and biological activities of medicinal mushrooms, the aim of this research was to examine the possibility of using edible *L. edodes* and non-edible *C. versicolor* medicinal mushrooms, for developing new kombucha products, in order to increase their consumption. To determine the possibility to perform kombucha fermentation, using novel mushroom-based substrates, it was necessary to monitor and compare the fermentation dynamics through determination of microbiological, physicochemical and chemical changes in fermenting kombucha broths. The cytotoxicity and immunomodulatory effects of novel kombucha polysaccharides isolated from kombucha samples prepared for consumption were investigated in human peripheral blood mononuclear cell cultures (PBMC) to examine its functionality and potential use as functional beverages or nutraceuticals. To the best of our knowledge, this is the first report showing that *C. versicolor* and *L. edodes* medicinal mushrooms can be used for kombucha fermentation processes. Additionally, this is also the first report of obtaining potentially novel crude polysaccharide nutraceuticals with immunomodulatory effects, after kombucha fermentations.

## 2. Material and methods

### 2.1. Cultivation of *C. versicolor* and *L. edodes* mushroom fruiting bodies

*Coriolus versicolor* basidiomycete mycelium originated from Košutnjak locust in Belgrade (Serbia) and was deposited in culture collection of the Department of Industrial Microbiology, Faculty of Agriculture, University of Belgrade (Serbia), while the mycelium of *L. edodes* (M 3776 species) originated from producer „Mycelia bvba" (Deinze, Belgium). Mycelium of both mushrooms were grown on wheat grains and used as inoculum for fruiting bodies production. The substrate for *C. versicolor* fruiting bodies production was prepared as previously described by Matijašević et al. (2016) and consisted of oak sawdust, wheat straw and wheat bran in the ratio 5:3:2. Fruiting bodies of *L. edodes* mushroom were cultivated on the substrate, which was prepared from the 65 % (w/w) of mixed sawdust from oak, beech and poplar (in 6:2:2 ratio), 25 % (w/w) of wheat bran, 8 % (w/w) of wheat straw and 2 % (w/w) of $CaSO_4$ x $2H_2O$. The substrates were humidified to 60 – 70 % (v/w), packed in polypropylene bags (Mycelia, Sac $O_2$, Microsac, Deinze, Belgium) and sterilized at 121˚C for 2 h (Badham, 1991). Inoculation of cooled substrates was performed in aseptic conditions by adding 10 % (w/w) of the inoculum. The mycelium colonisation of the substrates was done in the dark at 23 ± 2 °C, for fifteen to twenty days. For initiation of mushrooms fructification phase, bags were opened and placed at 18 ± 2 °C, 80 - 90 % of relative humidity and 500 -1000 lux of luminous intensity for 12 h/day. Fruiting bodies of mushrooms were collected, air-dried at 40 °C and ground to fine powder using laboratory blender.

### 2.2. Kombucha fermentation conditions

Two separate substrates for kombucha fermentations were prepared from each mushrooms' fruiting bodies. Mushroom powders (25 g) were mixed with 1 L of distilled water and extracted at 121 ˚C, 1.2 bars for 45 min. After the filtration through the cheesecloth, 300 mL of the filtrate was poured into 0.72 L of glass bioreactors, sucrose (70 g/L) was added and bioreactors were covered with cheesecloth.

Kombucha inoculum originated from the Department of Industrial Microbiology, Faculty of Agriculture, University of Belgrade (Serbia). Inoculation was performed by adding 10 % (v/v) of actively fermenting black tea (*Camellia sinensis* L.) kombucha broth. *C. versicolor* and *L. edodes* kombucha fermentations were conducted at 24 ± 1 °C in the dark and processes were monitored for eleven days (Sknepnek et al. 2018).

### 2.3. Monitoring of total number of microorganisms, total acids and pH during the kombucha fermentations

During the monitored period of eleven days, sampling was done from *L. edodes* (LE) and *C. versicolo*r (CV) fermenting broths. Samples were taken only once from a single bioreactor, on days 0, 1, 2, 3, 5, 7, 9 and 11 in triplicates. Frequent sampling may change the fermenting volumes, leading to different fermenting conditions over time. During this period, monitoring included determination of total number of yeasts and acetic acid bacteria (AAB), total acids and pH.

The number of yeasts was established on malt agar (Biolife, Milan, Italy) supplemented with chloramphenicol (50 g/L, Bioanalyse, Ankara, Turkey). The number of AAB was determined as described by Sknepnek et al. (2018), using YPM agar supplemented with cycloheximide (AppliChem GmbH, Darmstadt, Germany, 10 mg/mL) and penicillin (Bioanalyse, 20 mg/mL).

pH of the solution was monitored by pH meter (Basic 20, Crison, Barcelona, Spain), whereas, total acidity was determined by using 0.1M NaOH and phenolphthalein as an indicator (Velićanski et al., 2014).

### 2.4. Monitoring of sucrose, glucose, fructose and ethanol content during the kombucha fermentations by HPLC analysis

During the fermentation period of eleven days, samples of fermenting broths were taken six times (on days 0, 1, 2, 3, 7 and 11) in triplicates, to perform HPLC analysis. The quantitative analysis of sucrose, glucose, fructose and ethanol content were performed using Dionex Ultimate 3000 HPLC system (Thermo Scientific, Waltham, USA). In order to avoid contamination and changes in fermentation conditions, samples were taken only once from a single bioreactor (Duvnjak et al., 2016).

### 2.5. Polysaccharides isolation from *L. edodes* and *C. versicolor* kombucha beverages

After the desired acidity (3 g/L) was achieved, the fermentation process was terminated (Sknepnek et al., 2018). Formed cellulosic pellicle was discarded and the supernatant was centrifuged (Eppendorf 5840R, Hamburg, Germany) at 4000 *g* for 10 min. Evaporation was conducted at 40 °C using R-II rotavapor (Buchi Labortechnik, Flawil, Switzerland) to 10 % of the initial volume. After mixing of evaporated substrate and ethanol (96 % v/v) in ratio 2:1, polysaccharides were precipitated during 24 h at 4 °C. The samples were centrifuged for 10 min at 4000 *g* (Eppendorf 5840R, Hamburg, Germany) and pellets were washed in ethanol solution (70 % v/v). *L. edodes* (LEex) and *C. versicolor* (CVex) crude polysaccharides kombucha extracts were dried at reduced pressure and stored at 4 ˚C until further use (Kozarski et al. 2012).

### 2.6. Qualitative chemical analysis of novel polysaccharide kombucha extracts

Chemical properties of kombucha extracts (LEex and CVex) were analysed using an ATR-FTIR spectrophotometer (IRAffinity, Shimadzu, Kyoto, Japan). The resolution was 4 $cm^{-1}$ and spectra were recorded in the spectral range 4000 - 600 $cm^{-1}$ (Matijašević et al., 2016).

### 2.7. Quantitative chemical analysis of novel kombucha samples and their polysaccharide extracts

Quantification of total polysaccharides, phenols and flavonoids in kombucha samples and their polysaccharide extracts was done using UV-Vis spectrophotometer (UV-1800 Shimadzu). Total polysaccharides were determined at 490 nm and by applying D-glucose for standard curve (Dubois, Gilles, Hamitton, Reders & Smith, 1956). Total phenols were determined at 750 nm, using Folin-Ciocalteu reagent and gallic acid (Sigma Chemical Co., St. Louis, MO, USA) as standard, while for total flavonoids absorbance was measured at 510 nm and (+) - catechin was used for standard curve (Matijašević et al., 2016; Sknepnek et al., 2018).

### 2.8. Immunomodulatory and cytotoxic activity of polysaccharide kombucha extracts

#### 2.8.1. Cell cultures

All experiments involving human blood samples were approved by the Ethical Board of the Institute for the Application of Nuclear Energy (INEP). Peripheral blood mononuclear cells (PBMC) were obtained from buffy coats of healthy volunteers, who signed the Informed Consent in accordance with the Declaration of Helsinki, using density gradient centrifugation on lymphocyte separation medium 1077 (PAA, Linz, Austria).

To test the cytotoxicity of polysaccharide kombucha extracts PBMC ($3 \times 10^5$ per well of 96-well plate) were cultivated in complete RPMI 1670 medium (Sigma) containing 10 % fetal calf serum (FCS, Capricorn), 50 μM 2-mercaptoethanol (2-ME, Sigma), 1 % antibiotics (gentamicin, penicillin, streptomycin, ICN Galenika) in the presence or absence of different doses (7 - 500 μg/mL) of kombucha extracts for 24 and 48 h. Different doses of kombucha extracts were prepared in sterile PBS, and the pH was set to 7.2. After the cultures, the metabolic activity, corresponding to a total number of viable cells, was determined in MTT assay. To test whether kombucha extracts can modulate the proliferation and cytokines production by PBMC, the cells ($3x10^5$ per well of 96-well plate) were stimulated with polyclonal stimulator phytohemagglutinin (PHA, 10 μg/mL, Sigma) alone, or PHA and different doses

of kombucha extracts (7 - 500 μg/mL), for total of 48 h. After the cultures, cell-culture supernatants were collected for cytokines analysis, whereas the metabolic activity of total cells was determined in MTT assay.

2.8.2. MTT assay

To determine metabolic activity, PBMC culture supernatants were carefully removed and the solution of 3-[4,5 dimethyl-thiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT, Sigma, 50 μg/mL in PBS), was added to each well and the cultures were incubated for the next 4 h at 37 ˚C and 5 % $CO_2$. After that, the cell culture plates were centrifuged (350 *g* 10 min) and 0,1 mL of isopropyl alcohol was added to each well to dissolve formazan formed under the activity of cell succinate dehydrogenase. The absorbance (optical density, OD) was read at 570/650 nm (ELISA reader, Behring II, Heidelberg, Germany). All examinations were carried out in fourplicates. The results were expressed as the relative metabolic activity compared to the corresponding control cell (non-treated PBMC, 100 %).

Relative metabolic activity (%) = (OD of PBMC with polysaccharide kombucha extracts - OD corresponding polysaccharide kombucha extracts without cells) / (OD of control PBMC cultivated alone - OD of medium alone) x 100.

2.8.3. Cytokines detection

To assess the effect of kombucha extracts on cytokines production, the cell-free supernatants were collected from PHA-PBMC cultures after 48 h and stored at - 80˚C for cytokine analysis. The levels of IL-1β, IL-2, IL-4, IL-5, IFN-γ, IL-6, IL-8, IL-10 and TNF-α were determined from 50 μl of supernatants using immunobeads technology (BioLegend, San Diego, USA) and analysed by flow cytometer (BD LSR II). The unknown concentrations of cytokines were determined by constructing a dose-dependent curve from known standard concentrations of corresponding recombinant cytokines, according to manufacturer's protocol. To standardize concentrations of cytokines to the same number of viable cells

in cultures, the measured concentrations of cytokines were divided by index of metabolic activity in those cutlures, as a measure of total viable cells cultures.

Index of metabolic activity = OD of PHA-PBMC with polysaccharide kombucha extracts - OD corresponding polysaccharide kombucha extracts without cells) / (OD of control PHA-PBMC cultivated alone - OD of medium alone).

### 2.9. Statistical analysis

All experiments were repeated three times, and each experiment for monitoring of microbial, physicochemical and chemical changes were conducted in threeplicate, while the immunomodulatory and cytotoxic activities were determined in fourplicate. Statistical analysis was performed using GraphPad Prism software (GraphPad, La Jolla, CA, USA). The results are displayed as mean ± SD of three independent experiments and analysed using one-way analysis of variance (ANOVA), while the statistical significance ($p \leq 0.05$) between different treatment groups was determined using Tukey`s honesty significant difference (HSD) for $p \leq 0.05$. The correlation coefficient between total acid content and acetic acid bacteria number was calculated using linear regression analysis.

## 3. Results and discussion

### 3.1. Monitoring of total number of microorganisms, total acids and pH during the kombucha fermentations

To better understand of kombucha fermentation process in LE and CV kombucha samples, the changes of yeasts and AAB number **(Fig. 1A**), as well as the changes in total acids and pH (**Fig. 1B**) were monitored. The most intensive change of yeast cells number was detected 24 h after the beginning of fermentation. The maximal number was reached on the 2nd day, in LE kombucha (7.83 ± 0.01 log CFU/mL), followed by a constant decrease afterwards. The maximal number of yeasts in CV kombucha

reached 7.50 ± 0.02 log CFU/mL at the third day of fermentation, while the number under 7 log CFU/mL was determined after eleven days. The most intensive increase of AAB cells was detected after the first 24 h of fermentation, in both kombucha broths, but this change was less intense compared to the yeast cells increment. The maximal number (7.56 ± 0.01 log CFU/mL) of AAB cells was detected in CV kombucha after three days of fermentation, followed by a constant decrease in number afterwards. Regarding the LE kombucha, the maximal number (7.18 ± 0.06 log CFU/mL) was reached after two days, followed by a decrease until the fifth day. Repeated increase was detected on the 7th day, followed by continuous decrease in cell number until the end of monitoring period.

The obtained results are in accordance with previously published data. Namely, the maximal number of yeasts found during the fermentation of kombucha on traditional black or green tea substrates, ranged between 7 and 8 log CFU/mL (Chen & Liu, 2000; Sreeramulu, Zhu & Knol, 2000; Jayabalan, Marimuthu & Swaminathan, 2007; Neffe-Skocińska, Sionek, Ścibisz & Kołożyn-Krajewska, 2017). On the other hand, the data showed much higher variations of the maximal number of AAB cells in traditional kombucha production. Chen and Liu (2000) determined maximal number between 3 - 4 log CFU/mL during fourteen days of fermentation, while in other study, the number of AAB cells reached 7.61 log CFU/mL after ten days of fermentation (Neffe-Skocińska et al., 2017) Various factors can influence the variations in the number of microorganisms. The sensitivity of bacteria/yeast cells to low pH values and high concentrations of acids lead to decrease in cell number. Additionally, low concentration of accessible oxygen, due to carbon dioxide accumulation between cellulosic pellicle and fermentation broth was shown to influence their decrease (Chen & Liu, 2000). The observed reduction of AAB cells number in LE and CV kombucha samples probably occurred due to acid shock, while subsequent number increase in LE kombucha is most likely due to the propagation of bacterial strains resistant to high acids concentrations (Sreeramulu et al., 2000).

Total acidity increased during the monitored period (**Fig. 1B**). The maximal concentrations of 23.4 ± 2.4 and 33.5 ± 0.5 g/L were reached in LE and CV kombucha samples, respectively. The maximal change in LE kombucha was observed between the seventh and ninth day of fermentation (7.8 g/L), while the highest change in CV kombucha was detected between the 9th and 11th day (11.3 g/L). The regression analysis showed that there was no statistically significant correlation ($p \leq 0.05$) between total acids and AAB cells number, during the fermentation of LE and CV kombucha samples. Thus, we assume that the increase of total acids was most probably related to the survival and activity of specific AAB strains from kombucha inoculum. Compared to our results, previous data published for traditional substrates used in kombucha fermentations showed lower concentrations of total acids. Namely, total acids ranged from 1.15 and 9.5 g/L during the fermentations conducted between seven and fifteen days (Jayabalan et al., 2007; Malbaša, Lončar & Djurić, 2008; Velićanski et al., 2014). Only extended black tea kombucha fermentation led to higher acids concentrations, reaching 16.57 g/L of acetic acid and 27.02 g/L of titrable acidity, after twenty-one days (Chakravorty et al., 2016).

As a consequence of increasing content of acids, pH was decreasing in the fermenting kombucha samples. The initial pH of prepared LE substrate, before the inoculation, was 6.11 ± 0.05, while the pH of uninoculated CV substrate was 5.54 ± 0.03. The pH values decreased after the inoculation with kombucha inoculum, reaching the values of 5.39 ± 0.02 and 5.24 ± 0.01 in LE and CV substrates, respectively. The highest change in LE kombucha was detected in the first 24 h, reaching 1.37 pH units, while the change between the third and eleventh day was only 0.46 pH units. The lowest pH value in LE kombucha was detected at the end of monitored period (3.17 ± 0.03). In CV kombucha the highest change in pH was also detected in the first 24 h, reaching 1.27 pH units. A slower decrease was noticed after the third day of fermentation, while the change of only 0.33 pH units was detected in the last four days. During the same period, 26.6 g/L of total acids was synthesized in CV kombucha. At the end of monitored period, the pH value in this kombucha reached 2.97 ± 0.02. According to literature data, a decrease in

pH value is characteristic for black tea kombucha production at the beginning of fermentation (Sreeramulu et al., 2000; Malbaša et al., 2008; Velićanski et al., 2014). Despite the constant rise of acids in fermenting kombucha samples, a slower pH change occurs because of the buffer effect, *i.e.* the interaction between the synthesized acids and minerals or bicarbonate ions in the broth. That is the reason why total acids content should be taken into account for determination of fermentation process end point (Velićanski et al., 2014).

**Fig.1**

3.2. Monitoring of sucrose, glucose, fructose and ethanol content during the kombucha fermentations by HPLC analysis

Various microorganisms, present in symbiotic consortium of bacteria and yeasts (SCOBY), influence complex biochemical changes, which are still not completely understood in the kombucha fermentation process (Neffe-Skocińska et al., 2017). Yeasts' enzymes hydrolyse sucrose, which is mainly used as carbon source, into glucose and fructose. Yeasts are able to utilise both sugars and to create ethanol and carbon dioxide as dominant metabolites. Ethanol could be further oxidized by AAB into acetic acid, which is the most produced acid in kombucha beverage. Glucose is metabolised by certain AAB strains into organic acids, such as gluconic, or is utilised for the synthesis of cellulose,forming pellicle on the surface of the fermenting kombucha beverage. Beside yeasts, fructose is also metabolised by AAB into the acetic acid (Chen & Liu 2000; Sreeramulu et al., 2000). The change of sucrose, glucose, fructose and ethanol content in LE and CV kombucha samples prepared in this study is shown in **Table 1**. The content of sucrose decreased constantly in both samples and the most intensive reduction was observed between the first and the second day for LE kombucha. In both kombucha samples, statistically significant ($p \leq 0.05$) glucose content increment was observed on the 1st day, while complete utilisation of glucose was detected on the third day. Fructose concentration increment lasted for one day in LE

kombucha and two days in CV kombucha, and showed significant slower utilisation of fructose in CV kombucha,even the maximal number of AAB on the third day in CV kombucha was higher than in LE kombucha. Faster utilisation of fructose in LE kombucha is in accordance with higher number of yeasts (**Fig**. **1A**), which metabolise fructose in order to produce ethanol (Neffe-Skocińska et al., 2017). In line with this, higher maximal value of ethanol (4.3 % v/v) was determined in LE kombucha, although in both kombucha samples, ethanol content was increasing in the first days. Moreover, a higher intensity of sucrose reduction between the first and the second day, and the higher concentrations of ethanol on the third day in LE kombucha were most probably associated with the higher maximal number of yeast cells detected (7.83 log CFU/mL), as compared to the number of yeasts in CV kombucha. Additionally, AAB present in CV kombucha, expressed more intensive oxidative activity and more rapidly converted ethanol to acetic acid, which also had certain impact on lower concentration of ethanol (**Table 1**) and higher content of total acids (**Fig. 1A**) during the process. Lin et al. (2010) used hot water extracts from *L. edodes* mushroom's stipe and cane sugar in order to produce wine. They also determined intensive sucrose hydrolysis and the reduction of its concentration from 25 % to about 1 % in two days, which was followed by the increase in glucose and fructose and their utilization afterwards, depending on the *Saccharomyces cerevisiae* strain applied. In traditional black tea kombucha, the reduction of sucrose concentration was slower than in our study, while the reduction of glucose and fructose was not detected for ten (Lončar, Djurić, Malbaša, Kolarov & Klašnja, 2006) or sixty days (Chen & Liu, 2000). In the study of Chakravorty et al. (2016), significant production of reducing sugars was detected in the first seven days of fermentation, while their decrease was noted, afterwards. The same authors found the highest ethanol content of 0.28 g/L on the seventh day and lower concentrations thereafter (Chakravorty et al., 2016). According to the research conducted by Chen and Liu (2000), the highest ethanol content in traditional kombucha fermenting broth was reached between the tenth and the twelfth day of fermentation (0.55 % v/v) followed by its reduction, as well. Neffe-Skocińska et al. (2017) found the

maximal ethanol content of 11 g/L, which was constantly increasing for eleven days. In the present research, kombucha beverages produced using mushroom-based substrates contained higher concentrations of ethanol, compared to traditional kombucha beverages, as stated in the literature. Similarly, in the study of Talebi, Frink, Patil and Armstrong (2017), in eighteen tested commercial kombucha beverages, alcohol content ranged from 1.12 to 2.00 % (v/v).

It was previously reported that ethanol content in traditionally produced kombucha stimulated AAB to produce acetic acid (Chen & Liu, 2000). Accordingly, higher ethanol content in LE and CV kombucha samples led to the stimulation of certain AAB strains to produce acetic acid and faster rise of total acids in kombucha. Due to the fact that total acid content is the major parameter to determine fermentation end point, it can be concluded that using mushroom-based substrate leads to a shortage of kombucha fermentation process, compared to traditional black or green tea kombucha fermentation, as stated in the literature (Chakravorty et al., 2016; Neffe-Skocińska et al., 2017).

**Table 1.**

### 3.3. Qualitative chemical analysis of polysaccharide kombucha extracts

FTIR spectroscopy was used for the analysis of kombucha polysaccharide extracts composition, as a common and reliable method to this cause (Kozarski et al., 2012). The fermentation process of kombucha beverages was interrupted when total acids reached 3 g/L, followed by crude polysaccharides precipitation. The FTIR spectra of obtained samples are presented in **Fig. 2**. In both kombucha polysaccharide extracts from *L. edodes* (LEex) and *C. versicolor* (CVex), strong broad band were detected at ≈3300 $cm^{-1}$ indicating the presence of hydroxyl stretching vibrations, which indicate the presence of intra- and intermolecular interactions in polysaccharide chains. Additionally, these bands could also be connected with the asymmetric or symmetric N-H stretching in amino groups (Matijašević

et al., 2016). The band at 2930 $cm^{-1}$ refers to $CH_2$ stretching and banding vibrations of lipids (Ren, Hemar, Perera, Lewis, Krissansen & Bachanan, 2014). Absorption bands at ≈1415 $cm^{-1}$, ≈1250 $cm^{-1}$ and 3000 - 3500 $cm^{-1}$ indicate the presence of proteins and protein fractions (Ren et al., 2014; Sknepnek et al., 2018). The presence of OH groups, from phenolic components, was detected in region between 1410 and 1310 $cm^{-1}$ (Kozarski et al., 2012). Polysaccharides of various structure and content were detected in the middle infrared region, from 800 to 1200 $cm^{-1}$. These bands mostly originate from C-C and C-O stretching vibrations of glycosidic bonds and pyranose rings. Additionally, the presence of β-glycosidic bonds and β-glucans were indicated by the band identified at 1024 $cm^{-1}$, while the presence of α-glycosidic bonds were detected in the region 920 - 930 $cm^{-1}$. The bands detected only in the CVex sample were at: 860 $cm^{-1}$, corresponding to α-glucans, 987 $cm^{-1}$, most probably associated with CVex polysaccharides, and at 1650 $cm^{-1}$, asymmetric stretching vibrations of carbonyl (C=O) groups from polysaccharides (Ren et al., 2014). The band at 1627 $cm^{-1}$, solely found in LEex sample, indicates the presence of proteins (Meenu & Xu, 2019). Based on FTIR results, both samples were predominantly composed of polysaccharides, while the presence of phenols, lipids and proteins was also confirmed. Moreover, it is obvious that CVex sample possesses more complex polysaccharide structure, whereas LEex has more complex content of proteins.

**Fig. 2**

3.4. Quantitative chemical analysis of kombucha samples and their polysaccharide extracts

Quantitative chemical analysis of kombucha samples showed that the content of all tested compounds was significantly higher ($p \leq 0,05$) in CV kombucha, compared to LE kombucha (**Table 2**). Significantly higher content ($p \leq 0,05$) of total polysaccharides (745 ± 9,33 mg/g), phenols (27,13 ± 2,36 mg GAE/g) and flavonoids (3,37 ± 0,20 mg CE/g) was also found in CVex sample prepared from CV

kombucha, compared to LEex sample (**Table 2**). According to the literature data, in a dialyzed crude hot water extracts of *L. edodes* and *C. versicolor* mushrooms, Kozarski et al., (2012) detected 782 and 839 mg/g of total polysaccharide contents, respectively. A high content of total phenols in CVex sample was in accordance with previous findings (Siu et al., 2014). Choi, Lee, Chun, Lee and Lee (2006) showed that high temperature treatment (121˚C, 30 min) increased the total polyphenols concentration in *L. edodes* mushroom from 0,29 to 0,55 mg GAE/g. During the black tea kombucha fermentation process, Velićanski et al. (2014) reported the increase in polyphenol content from 0,35 to 0,57 mg GAE/mL. Moreover, Chakravorty et al. (2016) detected constant rise of total polyphenols from 1,5 to 2,25 mg GAE/g, during the twenty-one day of black tea kombucha fermentation. The same authors detected an increase of total flavonoids during the fermentation process (24 %), reaching the maximal concentration of around 15 μg quercetin equivalents per 100 mg, at the end of monitored period. Such an increase could be influenced by the enzymatic activity of microorganisms from kombucha inoculum, which led to the degradation of complex molecules causing the elevation of simpler compounds content (Chakravorty et al., 2016). The content of total flavonoids in CVex and LEex samples in our study was in accordance with previous research, when in eight edible mushrooms species the total flavonoid content between 0.9 and 3.0 mg CE/g was detected (Palacios et al., 2011). Due to the dominant presence of polysaccharides in kombucha samples (**Table 2**), we further tested cytocompatibility and immunomodulatory properties of polysaccharide extracts from CVex and LEex *in vitro*.

**Table 2.**

### 3.5. Cytocompatibility of novel polysaccharide kombucha extracts

Previous reports showed that various mushroom extracts, polysaccharides or polysaccharopeptides display immunomodulatory and anti-tumour activity (Siu et al., 2014). Kombucha extracts were also shown to possess beneficial anticancer properties (Jayabalan et al., 2011; Srihari, Arunkumar,

Arunakaran & Satyanarayana, 2013). To investigate whether novel kombucha extracts from *L. edodes* (LEex) and *C. versicolor* (CVex) also display immunomodulatory properties, dose-dependent effects of the extracts were studied in human PBMC cell cultures. To exclude the possibility that the differences in the production of cytokines by PBMC were due to cytotoxicity of kombucha extracts, cytotoxicity after 24 h (data not shown) and 48 h (**Fig. 3A**) in cell cultures by MTT was tested. LEex samples, tested in concentrations 7-500 µg/mL, did not influence the metabolic activity of PBMC significantly ($p \leq 0.05$) at both time points. On the other hand, a partial reduction of metabolic activity (13.3 %) was observed in 24 h- and 48 h- PBMC cultures with highest concentration (500 µg/ml) of CVex, compared to control untreated PBMC, but the difference was not significant statistically ($p \leq 0.05$) compared to control (**Fig. 3A**). According to ISO 10933-5, the reduction of MTT higher than 30 % is considered as cytotoxic (ISO, P., 2009). Therefore, it can be concluded that the cytotoxicity of kombucha extracts was not significant in human PBMC cell cultures.

To investigate whether kombucha extracts modulate PBMC proliferation, the cells were stimulated with a polyclonal stimulator PHA, either alone or in combination with different doses of kombucha (**Fig. 3B**), followed by measurement of metabolic activity, corresponding to total number of viable cells in culture. Thereby, kombucha extracts alone did not stimulate PBMC proliferation (**Fig 3A**). Compared to non-stimulated PBMC cultures, PHA-stimulated PBMC cultures displayed about 1.5-fold increase in metabolic activity after 48h cultures. CVex sample displayed stimulatory effects on PHA-induced proliferation of PBMC at doses 62,5 - 500 µg/mL (**Fig. 3B**). No further increase in MTT % was observed at doses higher than 125 µg/ml. LEex sample increased MTT% of PBMC cultures at 125 µg/mL, whereas other doses did not affect PHA-induced proliferation of PBMC. It was shown previously that PHA stimulates the proliferation of lymphocytes much more if PBMC contain antigen presenting cells, *i.e.* monocytes and dendritic cells, which do not proliferate upon PHA stimulation (Majumdar, Beachey, Tomai & Kotb, 1990). Therefore, it is possible that kombucha extracts acted on antigen presenting cells

to modulate proliferation in PBMC cultures, as well as directly on lymphocytes. To investigate how CVex and LEex modulate the production of cytokines, supernatants of PHA-PBMC cultures were used for cytokines measurements by flow cytometry immunobeads assay and ELISA.

**Fig. 3**

3.6. Cytokines detection

IL-2 is a crucial cytokine for the proliferation of lymphocytes, which is produced in an autocrine manner (Majumdar et al., 1990). The measurements of IL-2 concentrations in supernatants of PHA stimulated PBMC cultures treated with various concentrations of kombucha extracts confirmed that higher doses *L. edodes* (LEex) and *C. versicolor* (CVex) potentiate the proliferation of lymphocytes. As shown in **Fig. 4**, LEex stimulated IL-2 production when the highest dose was applied (500 μg $mL^{-1}$), while CVex stimulated its production at all tested doses (15 - 500 μg/mL), and similar phenomenon was observed when the levels of IL-2 were normalized to the total number of viable cells (**Supplementary Figure 1**). A stronger stimulatory effect of CVex on IL-2 production could explain its higher effect on the proliferation of PBMC, as compared to LEex. However, it should be taken into account that the measured concentrations of IL-2, in culture supernatants, are the result of IL-2 production and its simultaneous consumption by the proliferating lymphocytes. Therefore, the level of lymphocytes proliferation cannot be linked directly to the level of IL-2 in cell-culture supernatants. Moreover, additional cytokines may affect the proliferation of PBMC after the treatment with kombucha extracts, besides IL-2.

In order to determine immunomodulatory effects of kombucha polysaccharide extracts on immune cells functions, the analysis of proinflammatory cytokines (TNF-α, IL-6, IL-1β), chemokine (IL-8), Th1 cytokine (IFN-γ), Th2 cytokines (IL-4, IL-5) as well as anti-inflammatory cytokine (IL-10) was

analysed in supernatants of PHA-stimulated PBMC cultures (**Fig. 4**). LEex supressed the production of Th2 (IL-4 and IL-5) cytokines, as well as immunoregulatory cytokine (IL-10), at all tested concentrations and the lowest effect on IL-10 production was observed with the highest concentration of LEex applied (500 μg/mL). Similar phenomenon was observed when cytokine concentrations were normalized to the total number of viable cells in culture (**Supplementary Figure 1**). At the same time, LEex stimulated the production of proinflammatory cytokines TNF-α, IL-6 and IL-8, at the highest concentration applied (500 μg/mL). LEex extract did not modulate IFN-γ and IL-1β production by PHA-stimulated PBMCs. On the other hand, CVex stimulated the production of TNF-α (at 125 μg/mL) and IL-8 (at 125 μg/mL and 500 μg/mL), as shown in **Fig. 4**. This extract also displayed inhibitory effects on IL-10 production by PBMC at all doses tested, while there were no statistically significant effects on IFN-γ, IL-6 and IL-1β production. The strongest effect of both extracts was observed on the reduction of IL-4 and IL-5 cytokines production by PBMC. IL-4, IL-5 and IL-10, are key Th2 cytokines involved in development of allergies (Couper, Blount & Riley, 2008), so inhibition of their production could be beneficial for allergies suppression. In this context, it was previously reported that medicinal mushrooms can express anti-allergic activity (Badalyan, Barkhudaryan & Rapior, 2019), but the contribution of specific bioactive components was not elucidated. Here we showed for the first time, that novel kombucha polysaccharide extracts alone mediate such an effect on Th2 immune response. Further studies on specific kombucha's polysaccharides and other bioactive components *in vitro*, and *in vivo* on appropriate animal models of Th2 mediated pathologies (asthma, allergy, atopic dermatitis, etc.), are necessary to prove the hypothesis on antiallergenic effects and mechanisms of novel kombucha beverages and their components. Th2 cytokines, especially IL-10, were shown to inhibit the up-regulation of Th1 immune response and *vice versa* (Bretscher, 2019). However, we did not observe the up-regulation of IFN-γ in cultures with kombucha extracts, suggesting that additional signals are required for the up-regulation of Th1 immune response, a key mediator of an efficient anti-viral immune response (Snell, Osokine, Yamada, De la

Fuente, Elsaesser & Brooks, 2016). We have shown previously that endosomal toll-like receptor (TLR)3 and (TLR)7 agonists*, i.e*. poly (I:C) (Pavlović et al., 2015) and 7-thia-oxoguanosine (Čolić et al., 2014; Thorne, Tomić, Pavlović, Mihajlović, Džopalić & Čolić, 2015) respectively, as viral pathogen associated molecular patterns (vPAMPs) are excellent inducers of dendric cells-mediated induction of Th1 immune responses. In this context, it is possible that downregulation of Th1 inhibitors (Th2 cytokines and IL-10) could lower the activation threshold for the induction of Th1 response upon encountering a specific vPAMP, and thereby provide a more efficient immune response against viruses. This hypothesis deserves to be tested in appropriate *in vitro* and *in vivo* models in further studies.

Among kombucha polysaccharide extracts, CVex expressed a stronger stimulatory effect on proliferation of PBMC and the production of proinflammatory cytokines TNF-α and IL-8. IL-8 is produced exclusively by non-proliferating monocytes/macrophages (Matsuo, Sakai, Okubo & Yamaguchi, 2019). In contrast, both monocytes and Th1 cells produce TNF-α (Duque & Descoteaux, 2014; Furiati et al., 2019). When the measured levels TNF-α were normalized to the total number of viable PBMC in cultures, the stimulatory effect of CVex on TNF-α production was no longer present (**Supplementary Figure 1**), suggesting that the total increase in TNF-α levels was a consequence of increased number of TNF-α-producing lymphocytes in PBMC. However, additional studies on the effects of CVex on individual subsets of immune cells are necessary to prove this hypothesis. Different immunomodulatory effects of kombucha polysaccharides, could be due to their different chemical compositions as shown by FTIR analysis (**Fig. 2**) and spectrophotometry (**Table 2**). Probably, a more complex polysaccharide composition and a higher concentration of the main bioactive metabolites in CVex sample enabled its stronger effect on TNF-α and IL-8 stimulation by PBMC. Previously, Lee et al. (2006) showed that the duration of *C. versicolor* submerged mycelium culture cultivation and the synthesis of bioactive components, influenced the cytokine production in PHA stimulated PBMCs and anticancer potency of isolated polysaccharopeptide (PSP). The authors tested the activity of PSP (1

mg/mL) and found that mycelium cultivation from day zero to day ten, stimulated the production of IL-1β and TNF-α for 2,8- and 3,63-fold, respectively, while the stimulation of IFN-γ was weaker (Lee et al., 2006). Proteins detected only in LEex extract could be crucial for the stimulation of IL-6 production. TNF-α and IL-6 play important roles in local and systemic immune responses and organism's readiness to fight infections, while chemokine IL-8 is crucial for the attraction of leucocytes to the site of infection during the early stage of immune response (Matsuo et al.,, 2019). In addition, the inhibition of IL-10 production could be important for a more efficient proinflammatory immune response to potential infectious stimulants. Namely, it was shown that IL-10 suppress differentiation and function of Th1 and Th17 cells, antigen-presenting cells, as well as the production of proinflammatory cytokines (Couper et al., 2008). Therefore, an inhibited production of IL-10, in cultures treated with kombucha polysaccharide extracts (LEex and CVex) probably lowered the activation threshold for proliferation and production of proinflammatory cytokines such as TNF-α, IL-6 and IL-8. In order to elucidate the contribution to immunomodulatory effect of particular bioactive components from kombucha polysaccharide extracts, their isolation and purification require independent studies, which are currently in progress.

Fig. 4

## 4. Conclusions

The results presented in this paper revealed a great potential of *L. edodes* and *C. versicolor* medicinal mushrooms for using as a substrate in kombucha fermentation. Hot water extraction of produced mushroom fruiting bodies enabled the generation of substrate, which stimulated total acid production, and consequently provided shorter fermentation time. It was shown that kombucha products possess significant amounts of bioactive components, whose quantity depends on the applied mushroom. The anlysis of biological properties of kombucha polysaccharides suggested their highly desirable

immunomodulatory properties in human cell cultures. The obtained results on inhibitory effects of kombucha extracts on Th2 and IL-10 suggests that the consummation of kombucha beverages prepared on *L. edodes* and *C. versicolor* substrates, as well as consumption of isolated polysaccharide extracts, could be beneficial for prevention and treatment of Th2-mediated immunopathology such as allergic reactions, asthma or atopic dermatitis. Additionally, the stimulation of pro-inflammatory cytokines and reduction of anti-inflammatory IL-10 cytokine after the treatment of PHA stimulated PBMCs with kombucha mushroom polysaccharide extracts could contribute to the organism's defence potential against external pathogens such as viruses. Additional studies *in vitro*, and *in vivo* on appropriate animal models are necessary to further elucidate the contribution and mechanisms of different bioactive components of novel kombucha beverages. The promising results obtained on novel kombucha products, with valuable components and established bioactivity, support their recommendation as potential functional beverages or nutraceuticals.

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**Figures captions**

**Fig. 1. A:** Changes in acetic acid bacteria (AAB) number and yeasts number in *Lentinus edodes* (LE) and *Coriolus versicolor* (CV) kombucha samples. **B:** Changes of total acids and pH in *L. edodes* (LE) and *C. versicolor* (CV) kombucha samples. Results are expressed as mean value ± SD, from three independent experiments.

**Fig. 2.** Fourier transform infrared spectra of *Lentinus edodes* (LEex) and *Coriolus versicolor* (CVex) polysaccharide extracts from kombucha beverages.

**Fig. 3.** Cytotoxicity of polysaccharide kombucha extracts in human PBMC cultures. **A:** Cytotoxicity of polysaccharide kombucha extracts was tested in doses 7 to 500 μg/mL on human PBMC cultures ($3x10^5$/well of 96 wells plate) after 48 h of incubation by MTT assay. **B:** Proliferation of PBMC upon stimulation with PHA in the presence or absence of polysaccharide kombucha extracts (7 to 500 μg/mL) was determined using MTT assay after 48 h. K - control sample without applied extract, LEex - polysaccharide kombucha extract from *Lentinus edodes* medicinal mushroom, CVex - polysaccharide kombucha extract from *Coriolus versicolor* medicinal mushroom. Results are expressed as mean value ± SD from three independent experiments. *$p \leq 0.05$, compared to control or indicated by lines ($p \leq 0.05$).

**Fig. 4.** Cytokines production in PHA stimulated PBMC cultures after 48 h treatment with medicinal mushroom kombucha polysaccharide extracts immunobeads (all cytokines) assays and ELISA (IL-6, TNF-α), tested using ELISA assay. K- control sample without applied extract, LEex - polysaccharide kombucha extract from *Lentinus edodes*, CVex - polysaccharide kombucha extract from *Coriolus versicolor*. Results are expressed as mean value ± SD from three independent experiments, each carried out in 4-plicates, $*p \leq 0.05$, compared with the control or indicated by lines ($p \leq 0.05$).

**Table 1.** Concentration change of sucrose, glucose, fructose and ethanol during the fermentation of kombucha substrates prepared from *Lentinus edodes* (LE) and C*oriolus versicolor* (CV) medicinal mushrooms.

| | **Sucrose (g/L)** | | **Glucose (g/L)** | | **Fructose (g/L)** | | **Ethanol (%, v/v)** | |
|---|---|---|---|---|---|---|---|---|
| **Days** | **LE**[1] | **CV**[2,3] | **LE** | **CV** | **LE** | **CV** | **LE** | **CV** |
| **0** | 67.5±0.4$^{A}$ | 64.9±0.5$^{A}$ | 3.3±0.1$^{A}$ | 3.1±0.0$^{A}$ | 1.5±0.1$^{A}$ | 1.2±0.0$^{A}$ | 0.0±0.0$^{A}$ | 0.0±0.0$^{A}$ |
| **1** | 66.5±0.1$^{B}$ | 34.8±1.2$^{*B}$ | 5.8±0.1$^{B}$ | 3.5±0.1$^{\#B}$ | 6.5±0.1$^{B}$ | 4.6±0.2$^{\S B}$ | 1.7±0.0$^{B}$ | 1.3±0.1$^{\dagger B}$ |
| **2** | 6.1±0.5$^{C}$ | 13.2±1.1$^{*C}$ | 1.7±0.1$^{C}$ | 3.7±0.2$^{\#B}$ | 2.3±0.1$^{C}$ | 6.1±0.4$^{\S C}$ | 3.6±0.0$^{C}$ | 3.1±0.0$^{\dagger C}$ |
| **3** | 1.4±0.3$^{D}$ | 2.5±0.3$^{*D}$ | 0.0±0.0$^{D}$ | 0.0±0.0$^{C}$ | 0.7±0.1$^{D}$ | 4.8±0.1$^{\S B}$ | 4.3±0.1$^{D}$ | 2.1±0.1$^{\dagger D}$ |
| **7** | 0.6±0.0$^{E}$ | 1.0±0.1$^{E}$ | 0.0±0.0$^{D}$ | 0.0±0.0$^{C}$ | 0.0±0.0$^{E}$ | 3.5±0.2$^{\S E}$ | 4.0±0.0$^{E}$ | 2.5±0.2$^{\dagger D}$ |
| **11** | 0.6±0.0$^{E}$ | 1.1±0.0$^{E}$ | 0.0±0.0$^{D}$ | 0.0±0.0$^{C}$ | 0.0±0.0$^{E}$ | 0.0±0.0$^{F}$ | 3.5±0.1$^{C}$ | 0.4±0.2$^{\dagger E}$ |

[1]Results are expressed as mean value ± SD, from three independent experiments. [2]Mean values in the same row compared among LE and CV kombucha samples are significantly different if marked with * for sucrose, # for glucose, § for fructose and † for ethanol ($p \leq 0.05$, ANOVA, Tukey's HSD) [3]Means in the same column marked with different capital letters are significantly different ($p \leq 0.05$, ANOVA, Tukey's HSD).

**Table 2.** Content of total polysaccharide, total phenol and total flavonoid in kombucha samples from *Lentinus edodes* and *Coriolus versicolor* medicinal mushrooms and their polysaccharide extracts.

| Sample | Total polysaccharide content | Total phenol content | Total flavonoid content |
|---|---|---|---|
| | **(mg/mL)** | **(mg GAE/mL)** | **(mg CE/mL)** |
| **LE[1]** | $7.97 \pm 0.24^{A,2,3}$ | $0.19 \pm 0.02^{A}$ | $0.01 \pm 0.00^{A}$ |
| **CV** | $35.16 \pm 0.18^{B}$ | $0.33 \pm 0.01^{B}$ | $0.03 \pm 0.01^{B}$ |
| | **(mg/g)** | **(mg GAE/g)** | **(mg CE/g)** |
| **LEex** | $363.50 \pm 19.10^{a,4}$ | $2.37 \pm 0.67^{a}$ | $1.03 \pm 0.03^{a}$ |
| **CVex** | $745.00 \pm 9.33^{b}$ | $27.13 \pm 2.36^{b}$ | $3.37 \pm 0.20^{b}$ |

[1]LE – kombucha sample from *L. edodes*; CV – kombucha sample from *C. versicolor*; LEex - polysaccharide kombucha extract from *L. edodes*; CVex - polysaccharide kombucha extract from *C. versicolor*. [2]Results are expressed as mean value ± SD, from three independent experiments. [3]In the same column values marked with different capital letters are statistically analysed and are significantly different, $p \leq 0.05$, ANOVA, Tukey's HSD. [4]In the same column values marked with different small letters are statistically analysed and are significantly different, $p \leq 0.05$, ANOVA, Tukey's HSD.

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