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Proceeding Paper

Laetiporus sulphureus Mushroom Enhances Cytotoxic Effect of *Bifidobacterium animalis* spp. *lactis* on HCT-116 Cells in a Co-Culture System †

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Abstract: The study aimed to test the effect of probiotic *Bifidobacterium animalis* spp. *lactis* (BAL) on the HCT-116 cell line viability and to compare its effect with co-treatment BAL/*Laetiporus sulphureus* (EALS). The trypan blue staining method was used to estimate HCT-116 viability. The levels of NO_2^- were determined using 0.1% N-(1-naphthyl) ethylenediamine, as well as 1% sulfanilic acid. The determination of H_2O_2 was based on the oxidation of phenol red. Our results showed the significant cytotoxicity of the BAL on the HCT-116 cells in a co-culture system, while the BAL/EALS co-treatment further enhanced the cytotoxicity on the HCT-116 cells. We detected higher H_2O_2 and NO_2^- values in treatments with BAL, especially in the BAL/EALS co-treatment. The death of the HCT-116 cells may be due to elevated levels of H_2O_2 and NO_2^- and their products (peroxynitrites).

Keywords: edible mushroom; probiotics; *Bifidobacterium*; cancer cells; extract; H_2O_2; peroxynitrites

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1. Introduction

Colon cancer is one of the main health problems today. It is the second most common cancer diagnosed in women and the third most common in the male population [1].

Laetiporus sulphureus (Bull.) Murrill is an edible mushroom with sulfuric-yellow-colored fruiting bodies [2]. *L. sulphureus* is an important source of various bioactive components such as vitamins, carbohydrates, phenolic compounds, essential amino and fatty acids, and minerals. These components enable its biological activity [3]. It is known that certain components of mushrooms (such as polyphenols) interact with probiotics in the human colon due to their indigestion, and their mutual reaction is two-way.

Since the *L. sulphureus* mushroom has no cytotoxic effect on CRC cells [2,4], it is necessary to examine the combined anticancer effect of *L. sulphureus* and probiotics. *Bifidobacterium animalis* spp. *lactis* (BAL) is a normal inhabitant of the human colon and a widely used probiotic. Many studies show that certain species of the *Bifidobacterium* genus can induce an anticancer impact on colon cancer cells (Caco-2 and HT-29) [5].

The main aim of our study was to investigate the cytotoxic effect of BAL probiotics on the HCT-116 cell line and compare its effects with the BAL/EALS co-treatment. Also, we examined the levels of reactive species and compared them in both treatments. All experiments were performed in the modified HoxBan co-culture system.

2. Materials and Methods

Laetiporus sulphureus was gathered in the Adžine Livade at a height of 629 m (Šumadija region, Kragujevac, Serbia). The gathered mushroom material was dried in the air at room temperature. The 100 g of the mushroom fruiting body was soaked in 500 mL of ethyl acetate solvent. The finely dried ground mushroom material was macerated three times at room temperature every 48 h. Finally, the samples were filtered through sterile gauze, and the filtrates were evaporated to dryness using the evaporator (IKA, Sindelfingen, Germany). The dry ethyl acetate extracts (EALS) were stored at −18 °C before use [6].

Bifidobacterium animalis spp. *lactis* (BB-12) was obtained from the Microbiology Laboratory, Institute for Information Technologies, Serbia. The preparation of probiotic suspension was previously described in detail in a paper by Andrews et al. [7]. The turbidity of the initial suspension was measured using the McFarland densitometer (Biosan, Riga, Latvia).

The HCT-116 cell line was obtained from ATCC (Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle's minimal essential medium (DMEM) supplemented with fetal bovine serum and antibiotics. The HCT-116 cell viability was determined with trypan blue staining 12 and 24 h after incubation in the co-culture system [8].

The modified HoxBan co-culture system was formed in test tubes. The initial bacterial suspension contained about 10^8 colony-forming units (CFU/mL). Then, 1:4 dilutions of start suspension were additionally prepared in sterile 0.85% saline. To finish, 40 µL of BAL suspension was inoculated into 40 mL of sterile Mueller–Hinton soft medium. The coverslips with untreated cells were turned towards the agar surface without BAL (negative control (C_0)) or with BAL in agar (positive control (C)). In both negative and positive control, 10 mL of DMEM with 10% FBS and without antibiotics was added. HCT-116 cells on the surface of the co-culture system and BAL in the bacterial medium in the co-culture were overlaid with 10 mL of EARS treatment. The detailed procedure was described in a manuscript by Arsenijevic et al. [9].

The levels of NO_2^- were determined using 0.1% N-(1-naphthyl) ethylenediamine and 1% sulfanilic acid according to the protocol of Šeklić et al. [6]. On the other hand, determination of H_2O_2 was performed using horseradish peroxidase [10].

Statistical analyses were performed using the ANOVA method in statistical software SPSS (Windows, ver. 17, 2008, Chicago, IL, USA). $p < 0.05$ is considered a statistically significant difference (*#).

3. Results and Discussion

The significant cytotoxicity effect of the BAL on HCT-116 cell viability in a co-culture system was shown (Figure 1). Our results are in positive correlation with studies by Nowak et al. [11] and Faghfoori et al. [5]. These authors showed the cytotoxic effect of BAL on both HT-29 and Caco-2 cells. The Viability of HCT-116 cells was additionally decreased after incubation with the BAL/EALS co-treatment. It is especially noted at the EALS concentration of 50 µg/mL (Figure 1). Since the EALS extract had no cytotoxic activity, as shown by the results of Jovanovic et al. [4] and Younis et al. [2], we can conclude that noncytotoxic EALS extract enhances the cytotoxic effect on HCT-116 cells in co-culture with the BAL. The combined mechanism of action of BAL/EALS will be additionally investigated in future studies.

Cancer cells survive high levels of ROS and RNS by increasing their antioxidant status to optimize ROS/RNS-induced proliferation and, at the same time, avoid thresholds of these parameters that would trigger cell death [12,13]. However, disproportionately increased RNS and ROS levels can induce death in cancer cells. Our study demonstrated that the probiotic BAL caused an increase in H_2O_2 and NO_2^- levels in the HCT-116 after 24 h of incubation (Figure 2A). Through the incubation of the HCT-116 with BAL/EALS (10 and 50 µg/mL), the tested parameters were significantly increased, especially after 12 h (Figure 2B). These results indicate that BAL/EALS treatments caused acute oxidative stress in the HCT-116 cells. H_2O_2 and nitrites generate peroxynitrites. Then, the contact between peroxynitrites and residual H_2O_2 generates primary singlet oxygen. These molecules can cause local inactivation of protective catalase in cancer cells, and H_2O_2 and peroxynitrites survive at the site of local inactivation. This leads to the generation of secondary singlet oxygen through the interaction of H_2O_2 and peroxynitrites and catalase inactivation. Finally, the deactivation of catalases allows for the influx of H_2O_2 through aquaporins, leading to intracellular glutathione depletion and sensitization of the cancer cells for cell death induction through lipid peroxidation [14].

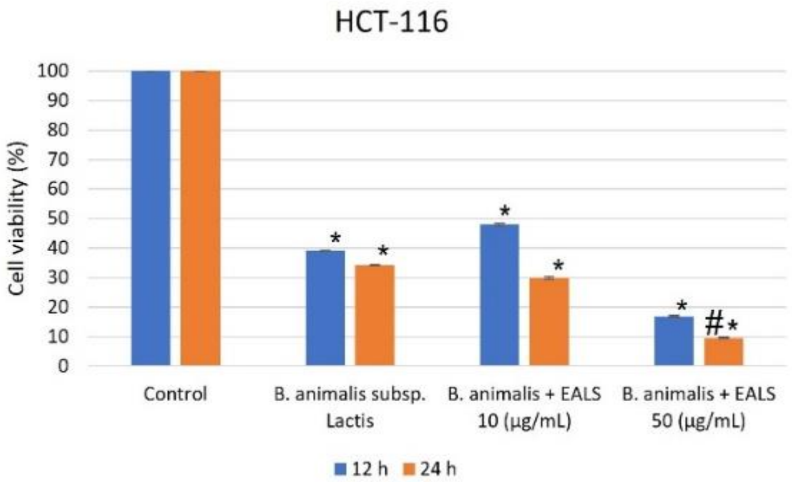

Figure 1. The percentage of the viable HCT-116 cells in the co-culture system after incubation with BAL and BAL/EALS (conc. 10 and 50 µg/mL). * A statistically significant difference between control and treatments at 12 and 24 h of incubation. # A statistically significant difference between BAL/EALS 10 µg/mL and BAL/EALS 50 µg/mL after 24 h.

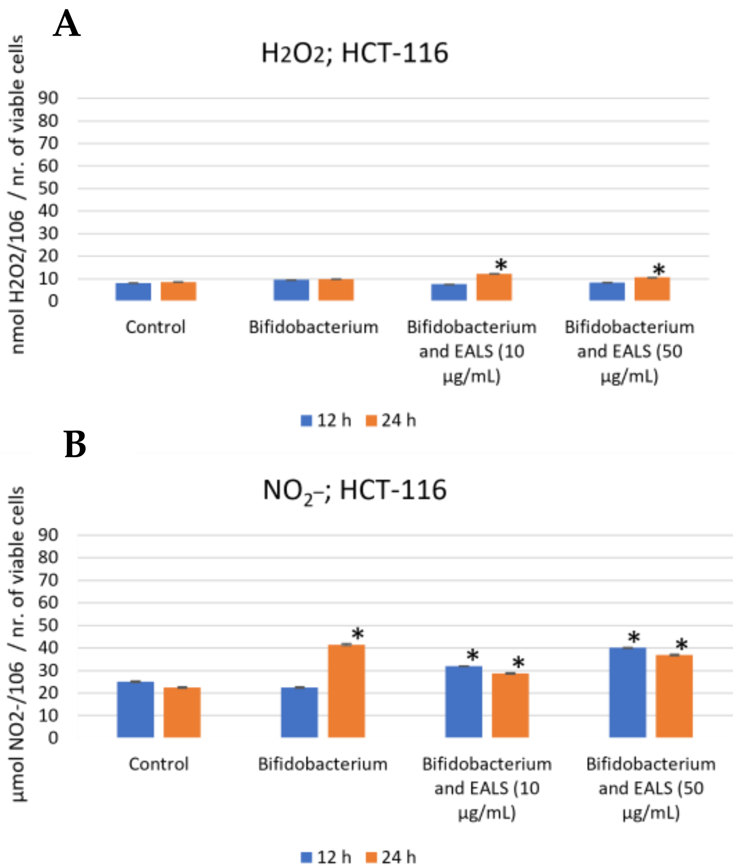

Figure 2. Levels of H_2O_2 (**A**) and NO_2^- (**B**) parameters in HCT-116 cell line after treatment with BAL and BAL/EALS (conc. 10 and 50 µg/mL). * A statistically significant difference between control and treatments at 12 h and 24 h.

4. Conclusions

Our study demonstrated the viability of colorectal cancer cells (HCT-116) after incubation with the probiotic BAL and compared its effects with co-treatment BAL/EALS. Our study demonstrated a significant reduction in HCT-116 viability in the co-culture system with BAL after 12 h of incubation. However, we observed that in the co-cultures of BAL and EALS extract, the viability of HCT-116 was further reduced. The strongest cytotoxic effect was noticed after incubation with BAL and EALS in a concentration of 50 µg/mL. The death of the HCT-116 cells is due to elevated levels of H_2O_2, NO_2^-, and their co-products (peroxynitrites).

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