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*Full Research Article*

# OXIDATIVE STATUS OF HONEY BEES INFECTED WITH *NOSEMA CERANAE* MICROSPORIDIUM AND SUPPLEMENTED WITH *AGARICUS BISPORUS* MUSHROOM EXTRACT

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**Abstract**

*Nosema ceranae,* a microsporidium species, is among the most common causes of bee diseases. The positive effect of *Agaricus bisporus* mushroom extract on the survival and immunity of *Nosema*-infected bees has been reported recently. The effect could be achieved by stimulating the expression of immune-related genes, but also by suppressing nosemosis. The aim of this work was to determine the effect of *A. bisporus* extract on the oxidative status of bees infected with *N. ceranae*. In a cage experiment on newly hatched bees, the effect of aqueous extract of champignon (*A. bisporus*, strain A15) was investigated. Six groups were formed: three groups were infected and received *A. bisporus* extract through food at different times (days 1, 3, and 6 after hatching), one group received the extract but was not infected (treatment control), one was only infected with *Nosema* (positive control) and one was neither infected nor received the extract (negative control). The effects were examined on samples taken on days 7 and 15 of the study. The activities of the antioxidant enzymes catalase (CAT), superoxide dismutase (SOD) and glutathione S-transferase (GST) and the concentrations of malondialdehyde (MDA) were determined. In comparison to the positive control, the

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enzyme activities and MDA concentrations were significantly lower in the groups fed with the mushroom extract supplement. In the negative control, the level of oxidative stress was lower than in the positive control. In comparison with the other groups, the values mostly did not differ significantly. The oxidative status of bees infected with *N. ceranae* was significantly better if they were fed with the *A. bisporus* extract.

**Key Words:** *Apis mellifera*, *Nosema ceranae*, *Agaricus bisporus*, oxidative stress, oxidative status

## INTRODUCTION

The honey bee (*Apis mellifera*) is of exceptional ecological and economic importance, primarily being a pollinator of numerous entomophilous plants (wild and cultivated). Moreover, honeybees produce honey, wax and other products, for which they are most often cultivated. Due to a large number of different factors, such as weather conditions and climate, inadequate nutrition, bad beekeeping practices with bee colonies, transport, various microorganisms and other stressors, bee colonies are prone to a wide array of diseases and colony collapse disorder (CCD, a phenomenon characterised by the absence of adult alive and dead bees in the hive, while relatively few workers and the queen remain (Stanimirovic et al., 2019), caused by multiple stressors).

One of the most widespread causative agents of bee diseases worldwide is the microsporidium *Nosema ceranae* (Martin-Hernandez et al., 2007; Klee et al., 2007; Higes et al., 2009; Higes et al., 2013). It produces spores, which are metabolically inactive forms and, thus, highly resistant. In performing various activities in the hive, the bees are orally infected, after which the spores reach the target tissues, i.e., the midgut cells. With the help of the filaments they possess, the spores penetrate new host cells and their sporoplasm is inserted into the cytoplasm. After the multiplication of the pathogen, spores are released from the damaged cells, continuing the invasion of not only the surrounding tissues but also other bees on leaving the host. The midgut of the bees is most severely affected (Fries, 2010), but research has shown that some other tissues and/or organs, such as the salivary and hypopharyngeal glands, ovaries (Steche, 1960; Sokolov and Grobov, 1963), the brain tissue (Gisder et al., 2010), and even the haemolymph (Glavinic et al., 2014) are not spared. Some research revealed that bees with nosemosis show increased hunger in comparison with their healthy counterparts, which implies energetic stress (Stanimirovic et al., 2019). Besides energetic stress, an increase in oxidative stress was detected in works monitoring the activity of antioxidative enzymes and the expression of genes coding for their synthesis (Dussaubat et al., 2012; Vidau et al., 2011).

Oxidative stress, the imbalance between oxidative and antioxidative compounds in the organism, is the state when the antioxidative mechanisms aimed at removing reactive oxygen species (ROS) are insufficiently efficacious. ROS have a role in overcoming pathogens in the body, play a signalling role between cells and fulfil various other useful roles. However, in high concentrations, they destroy the cells of the host organism

by reacting with many molecules, such as DNA and RNA, and cell structures, such as lipid membranes (Glavinic, 2019). Antioxidative enzymes fight against the ROS when needed. The activity of the antioxidative enzymes catalase (CAT), glutathione S-transferase (GST) and superoxide dismutase (SOD), and the concentration of malonyldialdehyde (MDA) are the indicators of oxidative stress that are most widely used in research (Liu et al., 2022).

The antibiotic used for the treatment of nosemosis is fumagillin. However, besides its anti-*Nosema* effect, its immunosuppressive effect on bees has also been proven (Glavinic et al., 2017, 2021a, b). In addition, antibiotic use today is associated with antimicrobial resistance, which can develop due to the frequent and/or inadequate use of antibiotics (Van den Heever et al., 2014; Glavinic et al., 2022). There are few countries in the world where fumagillin is registered for use, and it is worth looking for some alternatives in the fight against nosemosis. Many studies have been conducted and proved that dietary supplements can exert an immunostimulating and/or immunoprotective effect as well as other beneficial effects (Jovanovic et al., 2021; Stanimirovic et al., 2022). It has been demonstrated that adding vitamin C to the winter bee diet has some protective effect, particularly against diseases where oxidative stress is a major factor (Farjan et al., 2012), that caffeine can reduce the harmful effect of pesticides (Balieira et al., 2018), and that *Agaricus blazei* and *Agaricus bisporus* extracts increase the expression of most immune-related genes (Glavinic et al., 2021a, b). This work aimed to continue the research into the effects of the *A. bisporus* extract on bees infected with *N. ceranae*, by assessing its effect on their oxidative status.

## MATERIALS AND METHODS

All the bees in the experiment originated from healthy *A. mellifera* colonies living in the experimental apiary owned by the University of Belgrade, Faculty of Veterinary Medicine. The absence of *Nosema* infection in these was confirmed as described by Stevanovic et al. (2011, 2013). Using the methodology given by the *World Organisation for Animal Health* (WOAH) and the recommendations by COLOSS BEEBOOK (Fries et al., 2013), no other bee disease was detected, with the exception of *Varroa* infestation, which was kept at low levels.

### The tested preparations

The water extract of the common mushroom (*A. bisporus*, A15 strain) was prepared as described by Klaus et al. (2011). Briefly, dry fruiting bodies of the mushroom were subjected to extraction in distilled water at 121°C, at a pressure of 1.2 bar for 60 minutes. After filtration, the liquid was reduced to its 1/3 volume by evaporation and precipitated with 96% ethanol overnight. The material was centrifuged, dried at 40°C, ground into powder and stored in the refrigerator until further analysis. Besides polysaccharides (almost 70% including glucans), the extract contained proteins (5.31%) and phenolic compounds (2.7%) (Djekic et al., 2017; Vunduk et al., 2018).

## Bee hatching and experimental groups

Frames with capped bee brood (older capped brood – immediately before bee hatching) were taken from five healthy, strong colonies. In a very short time (less than five minutes, which was possible owing to the proximity of the apiary to the laboratory) the frames were transferred to the Laboratory for Animal Genetics at the Department of Biology (University of Belgrade, Faculty of Veterinary Medicine), where the experiment continued, and all the analyses were done. The frames were kept overnight to allow the emergence of as many bees as possible. The next morning according to methodology described by Glavinic et al. (2017), the newly hatched bees were placed in cages (60 bees per each cage) so as to form six groups in accordance with the experiment design shown in Table 1. The groups were as follows: the uninfected group – the negative control (C-), the group infected with *N. ceranae* spores on day 3 after hatching – the positive control (N), the group treated with *A. bisporus* extract – the treatment control (AB), and three groups infected with *N. ceranae* spores on day 3 after hatching and treated with the extracts from: day 1 after hatching (IAb1), day 3 after hatching (IAb3), and day 6 after hatching (IAb6). The experiment was performed in duplicate. From each cage, 5 worker bees were sampled on days 7 and 15 (in total 10 bees were used for each analysis per group per sampling moment). These were reared in an incubator at 34±1°C and a relative humidity of 66±1% for the following 15 days, in accordance with the sampling plan (Table 1). The bees were fed *ad libitum* on water-sugar syrup (ratio 1:1), and with the extract added to groups AB, IAb1, IAb3 and IAb6.

**Table 1.** Experimental design: groups of bees, days when the extract was applied, days when bees were infected with N. ceranae spores and days when samples were taken for analyses

| Group | Treatment with *Agaricus bisporus* started (from day) | Infection with *N. ceranae* spores (on day) | Sampling days | |
|---|---|---|---|---|
| C- | - | - | 7 | 15 |
| N | - | 3 | 7 | 15 |
| AB | 1 | - | 7 | 15 |
| IAb1 | 1 | 3 | 7 | 15 |
| IAb3 | 3 | 3 | 7 | 15 |
| IAb6 | 6 | 3 | 7 | 15 |

## Inoculum and artificial infection of bees

The abdomens of *N. ceranae*-infected bees were macerated in distilled water to obtain the spore solution. The presence of *N. ceranae* and the absence of *N. apis* were confirmed by the PCR method described by Martin-Hernandez et al. (2007). The number of spores was determined as described by Cantwell (1970). A fresh inoculum with a minimum of 99% viable spores (tested with a 4% trypan blue solution) was prepared

by mixing the spores with a 50% sucrose solution until the final concentration of 1 x $10^6$ spores/ml was achieved. Bees from the N, IAb1, IAb2 and IAb3 groups were infected with *N. ceranae* spore solution (inoculum) on day 3 after hatching according to the procedure described by Fries et al. (2013).

### Bee sampling

Bees were sampled from each group on days 7 and 15 of the experiment. Five bees were taken from each group for the analysis of oxidative stress parameters. The bees were taken out of the common cages individually with entomological tweezers by random selection. Dead or weak bees and those stuck in the syrup on which they were fed were excluded from the study.

### Measurement of oxidative stress parameters

To measure the oxidative stress parameters, the activities of antioxidative enzymes SOD, CAT and GST, as well as the concentrations of MDA were determined by the spectrophotometric analyses described in Dubovskiy et al. (2008) and adapted by Glavinic et al. (2021a; 2022). Briefly, 10% (w/v) homogenates (macerates) of whole bees in 100 mM Tris-HCl buffer (pH 7.4) were made, as described previously by Nikolic et al. (2015). Whole bees were homogenised in pestles using liquid nitrogen and mortars. The homogenates were centrifuged for 10 min at 10,000 g (4°C) in a refrigerated centrifuge (High-Speed Micro Centrifuge, GZ1730R GYROZEN) and the obtained supernatants were frozen at -20ºC until further analyses. All the analyses were done in duplicate on a UV/VIS Spectrophotometer BK-36 S390 (Biobase Bioindustry, Shanghai, China). The specific activities of the enzymes are given in the units of activity per mg of protein (U/mg of protein), and the MDA concentration in nmol/mg of protein.

### Statistical analysis

The survival of bees was monitored by the number of dead bees per day in each experimental group. The data on the survival distribution obtained in the Kaplan-Meier survival estimator were compared using the log-rank test. The data on all oxidative stress parameters were compared both between groups on the same day of sampling, and within each group between the two days. The data distribution was tested with the Shapiro-Wilk normality test. Data on the CAT, GST and SOD activities were normally distributed (Shapiro-Wilk test, $p>0.05$), unlike the concentrations of MDA, which were not (Shapiro-Wilk test, $p<0.05$). To obtain normality, the data on MDA were log transformed (Shapiro-Wilk test, $p>0.05$). To detect significant differences between groups, a two-way ANOVA was used, and the post hoc Tukey's test. All the results are presented as averages±standard deviations. The statistical analysis was done with GraphPad Prism 7 software (GraphPad, San Diego, CA, USA).

## RESULTS

### Bee survival

The number of dead bees (Figure 1) was significantly higher in the infected control group (N) compared to C-, AB, IAb1, IAb3 ($p<0.01$), and not significantly different ($p>0.05$) in comparison with group IAb6 (log-rank test).

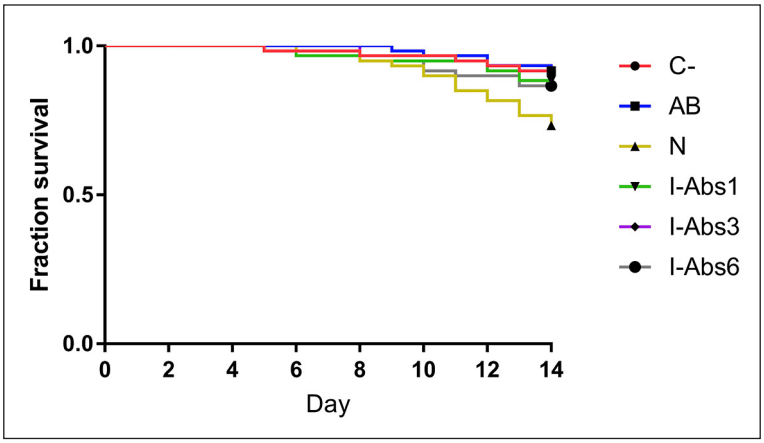


**Figure 1.** Survival of bees infected with *N. ceranae* (N), non-infected but treated with *A. bisporus* extract (AB), bees infected with *N. ceranae* and treated with *A. bisporus* extract from day 1 (group I-Abs1), day 3 (I-Abs3), and day 6 (I-Abs6), as well as bees from the control non-infected (C-) group.

### Catalase activity

There was a significant difference in CAT activity ($p<0.05$) within the AB group between the two sampling points: day 7 (21.83±2.91 U/mg) and day 15 (39.27±18.37 U/mg) (Figure 2). The comparison of CAT activity between the experimental groups

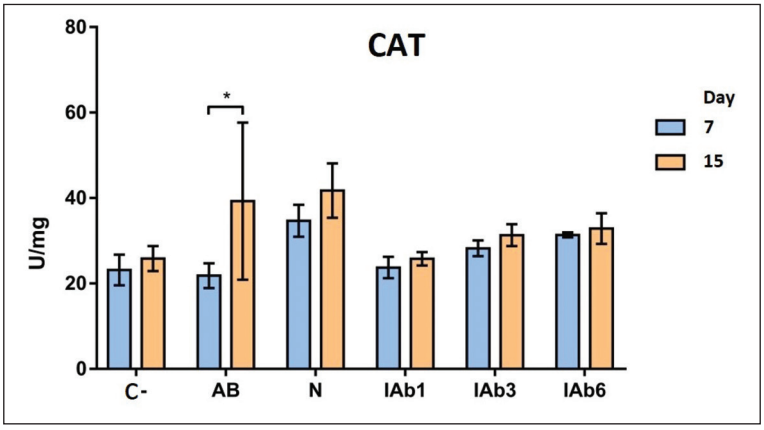


**Figure 2.** Comparison of CAT activity (units of enzyme per mg of total proteins) within each group between days 7 and 15

on day 7 detected significantly higher ($p<0.05$) values in N (34.67±3.75 U/mg) group in comparison with IAb1 (23.73±2.48 U/mg), C- (23.15±3.60 U/mg) and AB (21.83±2.91 U/mg) groups. On day 15, N (41.73±6.37 U/mg) group had significantly higher CAT activity than did IAb1 (25.76±1.56 U/mg) and C- (25.83±2.93 U/mg) groups. In addition, the activity of CAT was significantly higher in AB (39.27±18.37) group than in C- (25.83±2.93 U/mg) and IAb1 (25.76±1.56 U/mg) groups (Figure 3).

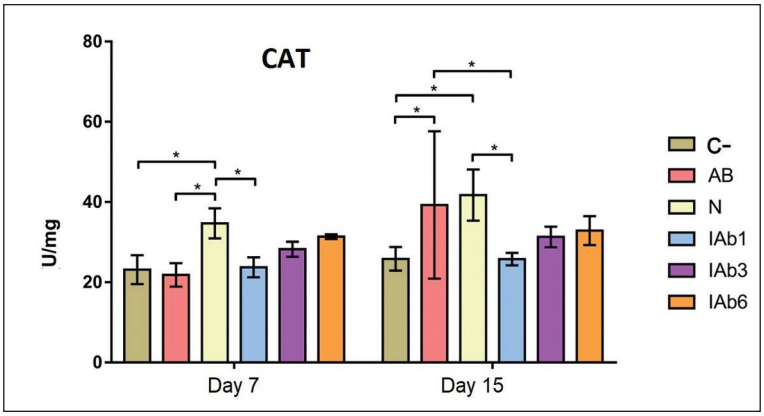


**Figure 3.** Comparison of CAT activity (units of enzyme per mg of total proteins) between groups on days 7 and 15

## Glutathione S-transferase activity

Only within the C- group did the analysis of GST activity reveal a significant ($p<0.05$) difference between the two sample times (250.10±35.83 U/mg vs. 186.00±37.39 U/mg), (Figure 4). On day 7, a significantly higher GST activity in N group (300.10±24.23 U/mg, $p<0.05$) was measured in comparison with IAb1 (237.70±28.43 U/mg), IAb3 (210.30±17.51 U/mg), AB (186.90±38.05 U/mg) and IAb6 (165.60±10.22 U/mg)

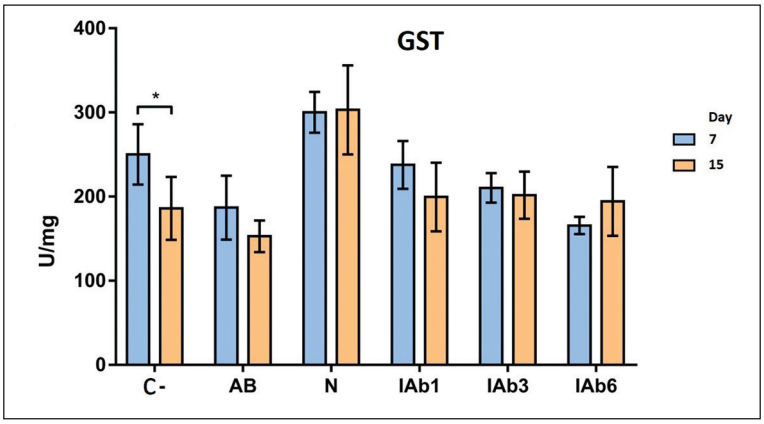


**Figure 4.** Comparison of GST activity (units of enzyme per mg of total proteins) within each group between days 7 and 15

groups. The IAb6 group had the lowest activity (165.60±10.22 U/mg), which was significantly ($p<0.05$) lower than the IAb1 (237.70±28.43 U/mg) and C- (250.10±35.83 U/mg) groups. GST activity was significantly higher in C- (250.10±35.83 U/mg) group than in AB (186.90±38.05 U/mg) group. On day 15, GST activity differed, group N (303.10±52.90 U/mg) had a significantly higher activity than did all the others: IAb3 (201.70±28.00 U/mg), IAb1 (199.50±40.82 U/mg), IAb6 (194.30±40.83 U/mg), C- (186.00±37.39 U/mg) and AB (152.80±18.88 U/mg), (Figure 5).

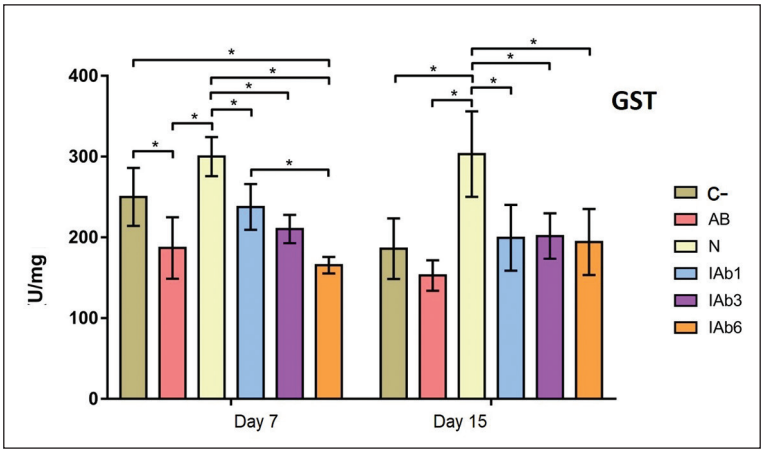


**Figure 5.** Comparison of GST activity (units of enzyme per mg of total proteins) between groups on days 7 and 15

## Malondialdehyde concentrations

The comparison of MDA concentrations between the two sampling times revealed a significant ($p<0.05$) difference only in N group (day 15 vs day 7: 1.22±0.16 nmol/mg vs 0.91±0.06 nmol/mg), (Figure 6). There were no significant differences between

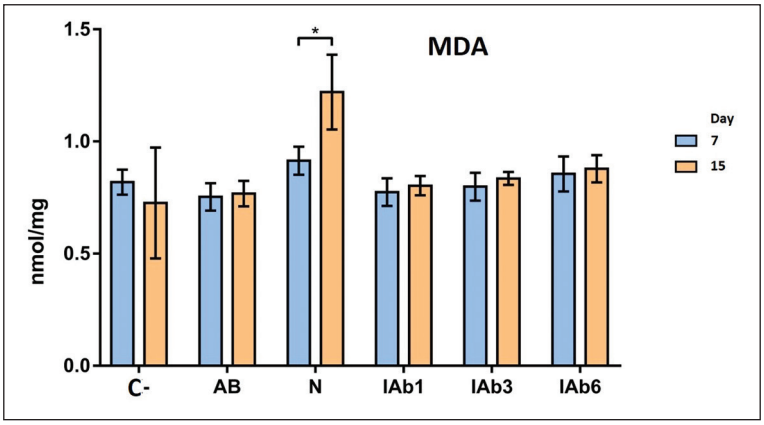


**Figure 6.** Comparison of MDA concentrations (nmol/mg of total proteins) within each group at two time points

groups (p>0.05) on day 7. On day 15, the MDA concentration was significantly (p<0.05) higher in N (1.22±0.17 nmol/mg) group in comparison with all the others: IAb1 (0.80±0.04 nmol/mg), IAb3 (0.84±0.03 nmol/mg), IAb6 (0.88±0.06 nmol/mg), AB (0.77±0.06 nmol/mg) and C- (0.73±0.25 nmol/mg) (Figure 7).

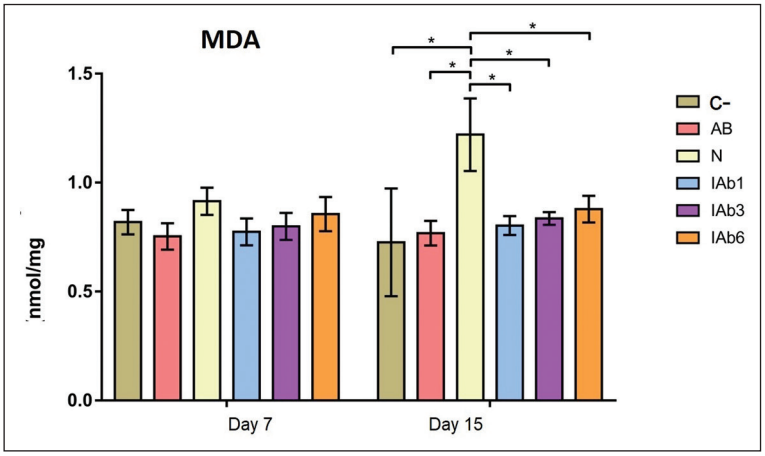


**Figure 7.** Comparison of MDA concentrations (nmol/mg of total proteins) between groups at each time point

## Superoxide dismutase activity

The activity of SOD did not differ significantly (p>0.05) between sampling days within any of the tested groups (Figure 8). On day 7, the SOD activity was significantly (p<0.05) higher in N group (52.71±16.35 U/mg) than in all the others: C- (32.32±6.02 U/mg), IAb6 (30.99±1.19 U/mg), IAb3 (30.54±2.01 U/mg), IAb1 (29.72±2.58 U/mg) and AB (29.03±7.61 U/mg). The relation remained similar on day 15: IAb6 (30.39±1.28 U/mg), IAb1 (29.66±1.20 U/mg), IAb3 (27.66±3.58 U/mg),

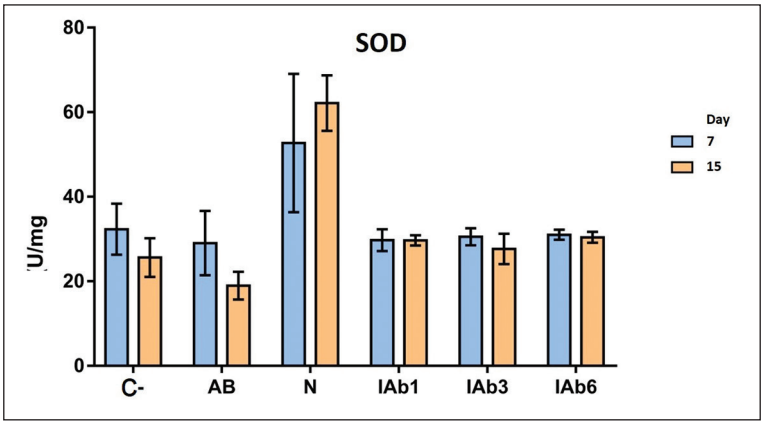


**Figure 8.** Comparison of SOD activity (units of enzyme per mg of total proteins) within each group between days 7 and 15

C- (25.61±4.56 U/mg) and AB. Moreover, at the second sampling time, AB group had significantly ($p<0.05$) lower SOD activity than did both IAb6 (30.39±1.28 U/mg) and IAb1 (29.66±1.20 U/mg) groups (Figure 9).

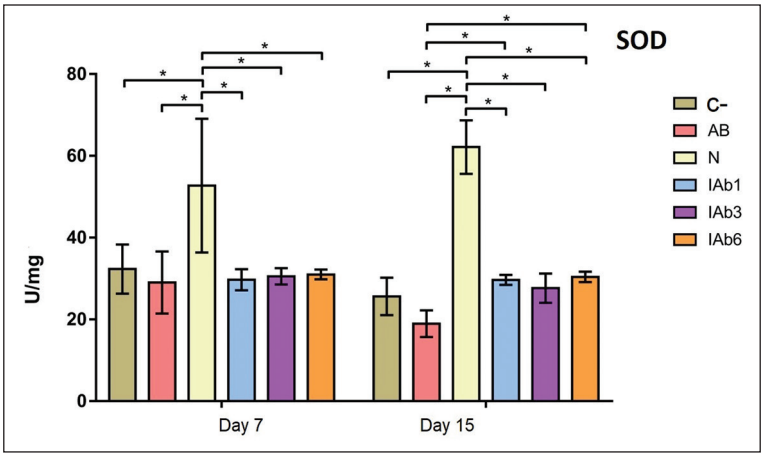


**Figure 9.** Comparison of SOD activity (units of enzyme per mg of total proteins) between groups on days 7 and 15

## DISCUSSION

It has been documented that some mushrooms are beneficent in reducing the oxidative stress in mammals (Wei and Griensven, 2008). In this research, we proved that the extract of *A. bisporus* mushroom added to the diet of *N. ceranae*-infected bees led to a significant decrease in mortality, similarly as Glavinić (2019) proved for *A. blazei*. Moreover, same authors revealed that *A. blazei* extract reduced *N. ceranae* spore loads, some parameters of oxidative stress and the downregulation of immune-related genes. Besides this fungus species, some effects of *A. bisporus* were investigated on *Nosema*-infected bees, i.e., the mortality of bees, spore loads and the expression of immune-related genes. However, oxidative stress has not been previously measured in bees that received the mushroom extract, so the current study provides this novel data. It was previously proven that *A. bisporus* reduces the mortality of bees, hinders the development of *Nosema* by inhibiting its adhesion to host cells, has some immunoprotective effects by increasing the expression of immune-related genes, especially in those bees that received the extract preventively or simultaneously with infection (Glavinic et al., 2021b). Given that the extract of *A. bisporus* acts immunoprotectively, and that another mushroom species, *A. blazei*, acts in the same way but is also an antioxidant, we hypothesised that *A. bisporus* could also have an antioxidant effect.

The CAT analysis on day 7 detected higher CAT activities in *Nosema*-infected bees, which could be associated with the oxidative stress caused by this endoparasite. Noticeably lower CAT activity was seen in groups C-, AB and IAb1, which indicates the absence of stress, i.e., the beneficial effect of the extract applied to bees from the

first day of the experiment (bees in groups AB and IAb1). This was expected, because in the work dealing with the effect of *A. bisporus* (Glavinic et al, 2021b) on the number of spores and gene expression, it was observed that the extract has the best effect when given preventively (from day 1) or simultaneously with the infection (day 3). However, the CAT activity increased significantly on day 15 in the non-infected group fed with *A. bisporus* extract. This finding was also not surprising given the results of a previous study on the effect of *A. blazei*: CAT activity increased on day 9 in comparison to day 6, which was followed by a decrease until day 15 (Glavinić, 2019). Another work that dealt with the effects of *A. blazei* on bees proved that the CAT activity was much lower in the treated control bees than that in the *Nosema*-infected ones (Glavinic et al. 2021a). This could be caused by β-glucans contained in *Agaricus* mushroom extract and their activity in prevention of microsporidian adherence to host cells (Roussel et al. 2015). However, this should be intensively investigated, having in mind that different stressors have been shown to induce oxidative stress (Morimoto et al., 2011; Simone-Finstrom et al., 2016; Chakrabarti et al., 2020).

There were large differences in GST activity between the tested groups. The activity was definitely highest at both time points in bees infected with *Nosema* and not treated at any time (N). This is in accordance with the results obtained with *A. blazei* (Glavinic et al., 2021a). Significantly lower activity, especially on day 15, was observed in all other groups, which indicates the antioxidant effect of the applied extract.

Regarding MDA concentrations, it was noticeable that on day 7, they did not differ between the groups. However, on day 15, there were significant differences. MDA was highest in N group (infected control), and much lower in all the others, which again favours the hypothesis that the *A. bisporus* extract exerted an antioxidant effect. Moreover, these results are consistent with the earlier obtained results for *A. blazei* (Glavinic et al., 2021a).

SOD activity did not differ significantly across time but did between the groups. The highest activity was detected in N group, significantly higher than in all other bee groups sampled on day 7. On day 15, however, the situation changed a little, and the extract failed to lower the enzyme activity that had been increased by the infection, while enzyme activity was lowered in the AB group, which was not infected but was fed the extract. These findings indicate the existence of an antioxidant effect.

When comparing only the groups that were infected and treated with the extract, despite the absence of significant differences, based on descriptive statistics, it is noticeable that the activity of CAT and the concentration of MDA on both days 7 and 15 were numerically the lowest in the IAb1 group, followed by IAb3, and IAb6. GST was lowest in IAb6 on day 7, slightly higher in IAb3, and then in IAb1 group, while on day 15, the levels of this enzyme were opposite. SOD concentrations were the lowest in IAb1 on day 7, in IAb3 on day 15, and the highest in IAb6 on both day 7 and 15. Thus, it can be concluded that the best effect was exactly in the group that was preventively fed with the extract (IAb1), followed by the group that received the extract simultaneously with the inoculation of *Nosema* spores (IAb3).

## CONCLUSION

The results of this research proved the antioxidative effects of the *A. bisporus* extract. The oxidative status of bees infected with *N. ceranae* was significantly better in groups fed with the mushroom extract. Thus, the extract seems to have a protective effect and has significant potential for combatting the cosmopolitan parasitic species, *N. ceranae.*

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**Authors contributions**

UG and ZS designed the study. DDZ and MR drafted the manuscript. UG, DDZ, SJ and MZ performed laboratory analyses, UG, MR, SJ and MZ performed data curation. UG, ZS, NA and MR interpreted the data. ZS, UG, MR and NA revised the manuscript and supervised the experiment.

**Competing interests**

The authors declare that they have no competing interests.

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## OKSIDATIVNI STATUS PČELA ZARAŽENIH MIKROSPORIDIJOM *NOSEMA CERANAE* I TRETIRANIH EKSTRAKTOM GLJIVE *AGARICUS BISPORUS*

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**Kratak sadržaj**

Među najučestalije izazivače oboljenja pčela spada mikrosporidija *Nosema ceranae*. Poznato je pozitivno dejstvo ekstrakta gljive Agaricus bisporus na preživljavanje i imunitet pčela inficiranih nozemom. To se postiže stimulacijom ekspresije gena značajnih za imunitet i suzbijanjem nozemoze. Cilj ovog rada bio je utvrditi kakav efekat ima ekstrakt šampinjona A. bisporus na oksidativni status pčela inficiranih mikrosporidijom *N. ceranae*. U kaveznom eksperimentu na novoizleženim pčelama ispitivan je efekat vodenog ekstrakta šampinjona (*A. bisporus*, soj A15). Oformljeno je šest grupa: tri grupe su bile inficirane i u hrani dobijale ekstrakt A. bisporus u različitim momentima (prvog, trećeg i šestog dana od izleganja), jedna grupa je dobijala ekstrakt, ali nije bila inficirana (kontrola tretmana), jedna je bila samo inficirana nozemom (pozitivna kontrola) i jedna nije bila inficirana niti je dobijala ekstrakt (negativna kontrola). Efekti su ispitivani na uzorcima uzetim 7. i 15. dana eksperimenta. Određene su aktivnosti antioksidativnih enzima katalaze (CAT), superoksid dismutaze (SOD) i glutation S-transferaze (GST), kao i koncentracija malonaldehida (MDA). Aktivnosti enzima i koncentracija malonaldehida bile su značajno niže kod grupa koje su dobijale ekstrakt šampinjona u poređenju sa pozitivnom kontrolom. Kod pčela iz negativne kontrole zabeležen je niži nivo oksidativnog stresa u odnosu na pozitivnu kontrolu, a u odnosu na druge grupe, vrednosti se uglavnom nisu nisu značajno razlikovale. Oksidativni

status pčela inficiranih mikrosporidijom *N. ceranae* bio je značajno bolji ukoliko su one prihranjivane ekstraktom gljive *A. bisporus*.

**Ključne reči:** *Apis mellifera*, *Nosema ceranae*, *Agaricus bisporus*, oksidativni status