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Leticia S. Ansaloni, Valentina Videčnik, Patrycja Staniszewska, Aneta Strachecka, Marija Gregori, Andrej Gregori, Caio E. C. Domingues & Aleš Gregorc

Fungal extracts influence lifespan and immune responses in the Carniolan honey bee (*Apis mellifera carnica*, Pollmann 1879)

Leticia S. Ansaloni[1,a,*], Valentina Videčnik[1,a], Patrycja Staniszewska[2], Aneta Strachecka[2], Marija Gregori[3], Andrej Gregori[3,4], Caio E. C. Domingues[1], Aleš Gregorc[1]

[1] University of Maribor, Faculty of Agriculture and Life Sciences, Pivola 10, 2311 Hoče, Slovenia

[2] Department of Invertebrate Ecophysiology and Experimental Biology, Faculty of Animal Sciences and Bioeconomy, University of Life Sciences in Lublin, 50A Doświadczalna Street, 20-280 Lublin, Poland

[3] MycoMedica Ltd., Podkoren 72, 4280 Kranjska Gora, Slovenia

[4] University of Ljubljana, Biotechnical Faculty, Jamnikarjeva 101, 1000 Ljubljana, Slovenia

[a] These authors contributed equally to this work
[*] **Corresponding author**: leticia.salvioni1@um.si

Abstract

Various stressors reduce the health of honey bees (*Apis mellifera*) and colony survival. Therefore, there is an ongoing search for substances that strengthen bee immunity. Fungal extracts, which exhibit antimicrobial and antioxidant properties and mitigate viral infections, may prove to be such substances. The aim of this study was to determine how fungal extracts from *Ganoderma lucidum* (GL), *Hericium erinaceus* (HE), *Inonotus obliquus* (IO), and *Trametes versicolor* (TV) affect Carniolan honey bee longevity, antioxidant activity (CAT, GPx, GST, SOD, and TAC levels) and metabolic marker activity (ALP, ALT, and AST). Haemolymph for biochemical analyses was collected from bees at 7, 14, and 21 days after exposure to fungal extracts and stored at –25°C until analysis. GL treatment increased bee longevity by 16.4% compared to untreated bees ($p < 0.0001$). Bees fed with fungal extracts showed increased activities of metabolic markers and antioxidants after 14 and 21 days compared to bees only fed sugar syrup ($p<0.0001$). Among all groups, bees fed IO had the highest levels of markers and antioxidants. This study demonstrates that

including fungal extracts in the bee diet improves immune indicators in bees.

Keywords: antioxidant enzymes, bee nutrition, haemolymph, insect immunity, fungi, metabolic markers

Introduction

The importance of honey bees as pollinators in natural habitats and for a wide variety of crops, as well as their relevance to the beekeeping sector, has been extensively documented[1-4]. However, Colony Collapse Disorder (CCD) has also been reported over the years, along with numerous factors that may be linked to it[5-9]. Among these stressors, nutritional stress can compromise immune responses and overall colony health[10,11]. According to Branchiccela[10], nutritional stress can affect colonies both in the short and long term. From this perspective, it is important for beekeepers to implement good management practices in order to keep the colonies healthy[12].

Honey bees, like other insects, have individual immunity, where physiological responses (cellular and humoral) work as a barrier to protect against diseases[13]. Within humoral immunity, biochemical defences such as the antioxidant system play a key role. Antioxidant enzymes provide protection against oxidative stress, for example, by neutralising harmful radicals and oxidants produced when bees encounter harmful factors, including anthropogenic factors, parasites, and pathogens[14,15]. These compounds include glutathione peroxidase (GPx), catalase (CAT), superoxide dismutase (SOD), and non-enzymatic antioxidants (e.g., glutathione, uric acid, urea, albumin, creatinine, and vitamins). Enzymes involved in toxin metabolism and detoxification include monooxygenase, glutathione S-transferase (GST), cytochrome P450 (P450), carboxylesterase (COE), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and gamma-glutamyl

transpeptidase (GGTP)[16-18]. ALP, AST, and ALT are also called metabolic markers or biomarkers. All these compounds work to capture free radicals and transform them into their non-toxic, less reactive forms. Total antioxidant capacity (TAC) is an indicator of the organism's overall antioxidant defence[19]. These compounds are synthesised in the fat body and then released into the haemolymph and distributed throughout the organism[20]. Furthermore, antioxidants can be obtained from food, both from pollen collected by the bees themselves[21-23] and from stimulants (e.g. vitamin C, CBD, CoQ-10, caffeine, curcumin, fish protein hydrolysate) administered to the colony by the beekeeper[24-32].

Hence, haemolymph plays an important role in insect immunity and homeostasis[33]. Its biochemical composition can provide valuable information regarding physiological status in honey bees[34] and is closely related to various factors the insects encounter, such as pesticides, diet type and quality, pathogens, and even the size of the cells in which they developed[35-39].

All of these dietary supplements and administered stimulants have short-term effects. Therefore, there is a constant search for new biostimulators with longer-term effects for use in beekeeping. In the same context, fungal extracts can be a promising natural source of bioactive compounds against pathogens, as well as a good alternative to improve colony health in honey bees[40-42].

It is well known that many honey bee diseases and parasites need to be regularly controlled using chemicals, which can lead to drug resistance after long-term use[43,44]. A sustainable way to keep honey bee colonies strong is by using fungal extracts. There is evidence of the potential of fungal extracts to reduce the microsporidium *Nosema ceranae*[42] and virus levels in honey bees such as deformed wing virus (DWV) and Lake Sinai virus (LSV)[40]. Additionally, entomopathogenic fungi have shown potential effectiveness against *Varroa destructor*[45]. Despite these findings, their effects on the antioxidant system activity of *A. m. carnica* (Pollmann, 1879) remain overlooked, and further studies are needed. Therefore, the aim of this study was to determine the effect of four different fungal extracts

(*Ganoderma lucidum* - GL, *Hericium erinaceus* - HE, *Inonotus obliquus* - IO, and *Trametes versicolor* - TV) on the survival rate, activities of metabolic markers (ALP, ALT, and AST) and antioxidant enzyme activities (CAT, GPx, GST, and SOD), and TAC levels in *A. m. carnica*.

Results

Survival rate

To assess survival rates, Carniolan honey bee foragers were fed fungal extracts *ad libitum* for 46 days. The results showed that the GL group was the only fungal extract to significantly increase longevity for bees (p=<0.001) (Fig. 1). The GL group presented a mean survival time of 25.890 ± 0.789 days (95% confidence interval bound = 24.343 - 27.436 days), representing a 16.39% increase in survival time when compared to the CTL group, which showed a mean survival time of 22.444 ± 0.839 days (95% confidence interval bound = 20.599 - 23.888 days) (p = 0.00584). The GL group also improved the survival time compared to the TV group (mean survival time of 21.500 ± 0.875 days; 95% confidence interval bound = 19.786 - 23.214 days), by 29.42% (p=0.00169), and the HE group (mean survival time of 21.077 ± 0.842 days; 95% confidence interval bound = 19.428 - 22.726 days) by 22.83% (p = 0.0000380).

The IO group, with a mean survival time of 24.608 ± 0.820 days; 95% confidence interval bound = 23.001 - 26.214 days, showed a 16.75% increase in survival time compared to the HE group (p = 0.0141). Although bees in the IO group had a 10.65% higher survival rate than those from the CTL group, no statistical difference was observed (p = 0.143). Similarly, the HE and TV groups had comparable results to bees from the CTL group (p = 0.986).

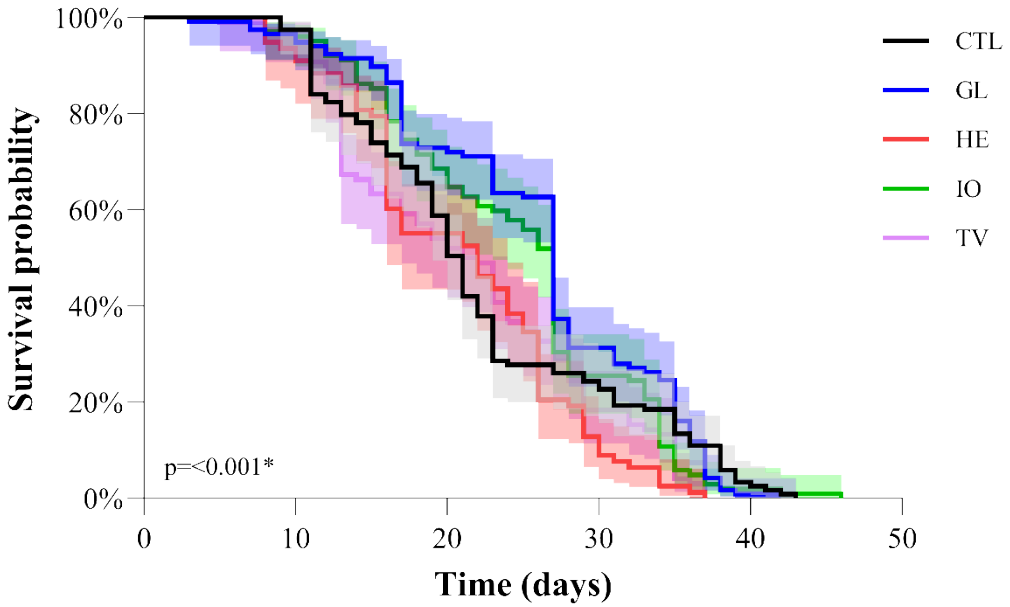

Figure 1. Survival probability of Carniolan honey bees after feeding on fungal extracts (**GL:** *G. lucidum*, **HE:** *H. erinaceus*, **IO:** *I. obliquus*, and **TV:** *T. versicolor*, each at a concentration of 4%), and syrup alone (**CTL**: control). n = 100 bees per experimental group. *GL showed a statistically significant difference (p = <0.001) to the CTL, HE, and TV groups. Survival analysis (Kaplan-Meier), chi square 26.10, df = 4, followed by multiple comparisons (Holm-Sidak method). Shaded areas represent 95% confidence intervals.

Metabolic markers

ALP activity in the haemolymph of bees from the GL (p = 0.0042), HE (p = 0.0014), and IO groups (p = 0.0042) was higher after 7 days compared to the CTL group (Fig. 2). No differences were observed between bees from the TV group (p = 0.2239) and the CTL group. The highest mean value after 7 days was recorded in bees from the HE group (6.557), while the CTL group demonstrated the lowest mean value (5.482). After 14 and 21 days, all the groups treated with fungal extracts exhibited higher ALP activity (p<0.0001) in comparison to bees from the CTL group, which recorded the lowest mean values (14 days: 10.930, and 21 days: 19.595). The IO group registered the highest mean values after 14 days (14.649) and 21 days (25.099).

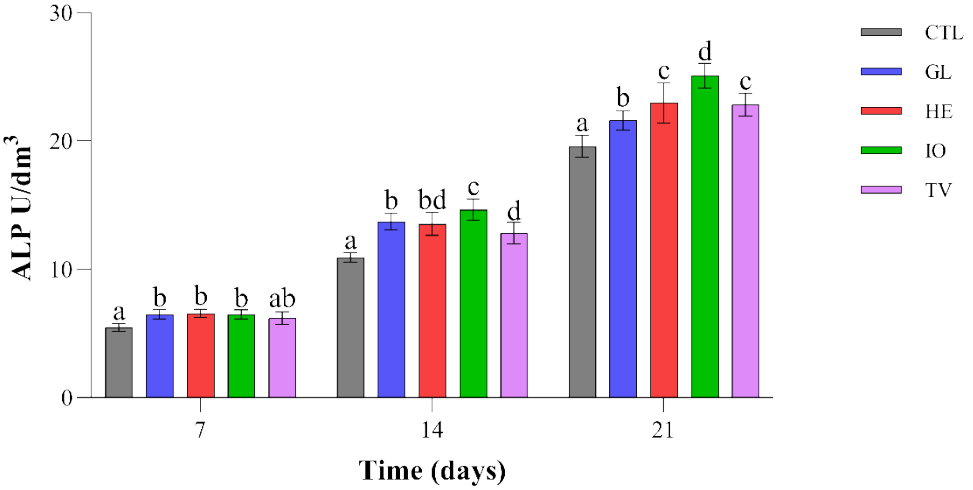

Figure 2. Alkaline phosphatase (ALP) activity in the haemolymph of Carniolan honey bees after 7, 14, and 21 days of feeding with fungal extracts (**GL:** *G. lucidum*, **HE:** *H. erinaceus*, **IO:** *I. obliquus*, and **TV:** *T. versicolor*, each at a concentration of 4%) and syrup alone (**CTL:** control). n = 20 bees per experimental group/time. Two-way ANOVA, interaction, F (8, 285) = 27.45, $p<0.0001$; time, F (2, 285) = 11046, $p<0.0001$; fungal extract, F (4, 285) = 153.2, $p<0.0001$. Statistically significant differences among treatments within each age group (7, 14, and 21 days) are indicated by letters. Data are presented as means ± standard deviation.

Regarding ALT activity in the haemolymph of bees, after 7 days only the IO group ($p = 0.0001$) showed a statistically significant difference compared to the CTL group (Fig. 3). The bees from the GL ($p = 0.9952$), HE ($p = 0.1792$), and TV groups ($p>0.9999$) were similar to the CTL group. In addition, the IO group had the highest mean value (25.229), and the TV group had the lowest mean value (23.415) after 7 days. After 14 and 21 days, all the fungal extract groups presented higher ALT activity ($p < 0.0001$) compared to the CTL group, which exhibited the lowest mean values (14 days: 30.745, and 21 days: 56.255). The IO group showed the highest mean values (14 days: 35.794, and 21 days: 63.199).

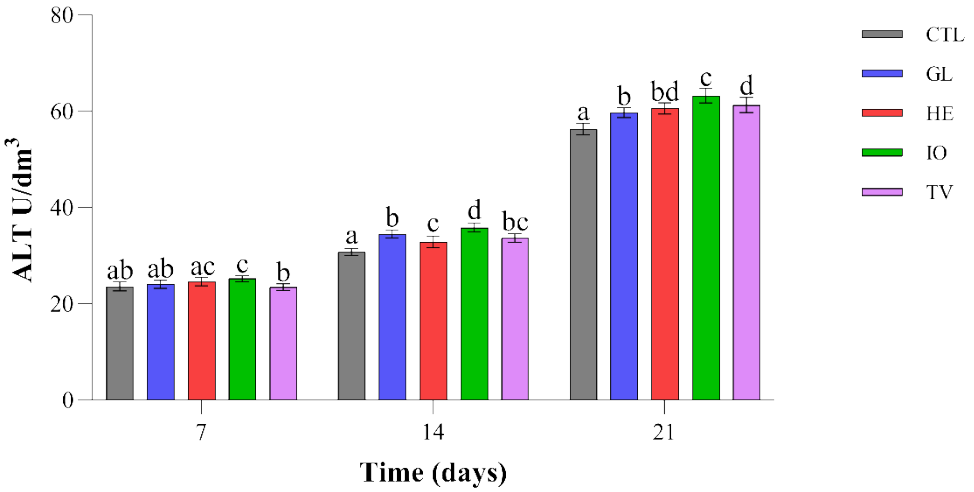

Figure 3. Alanine aminotransferase (ALT) activity in the haemolymph of Carniolan honey bees after 7, 14, and 21 days of feeding with fungal extracts (**GL:** *G. lucidum*, **HE:** *H. erinaceus*, **IO:** *I. obliquus*, and **TV:** *T. versicolor*, each at a concentration of 4%) and syrup alone (**CTL:** control). n = 20 bees per experimental group/time. Two-way ANOVA, interaction, F (8, 285) = 26.21, p<0.0001; time, F (2, 285) = 32232, p<0.0001; fungal extract, F (4, 285) = 143.7, p<0.0001. Statistically significant differences among treatments within each age group (7, 14, and 21 days) are indicated by letters. Data are presented as means ± standard deviation.

In relation to AST activity in the haemolymph of bees, after 7 days the GL (p>0.9999), HE (p = 0.9999), IO (p = 0.9912), and TV groups (p = 0.7563) showed no difference compared to the CTL group (Fig. 4). After 14 and 21 days, the GL (p<0.0001), HE (p<0.0001), IO (p<0.0001) and TV groups (p<0.0001) presented higher values of AST activity compared to the bees from the CTL group. The highest mean values were recorded in the IO group (14 days: 65.510, and 21 days: 98.047), while the CTL group showed the lowest mean values (14 days: 55.050, and 21 days: 84.811).

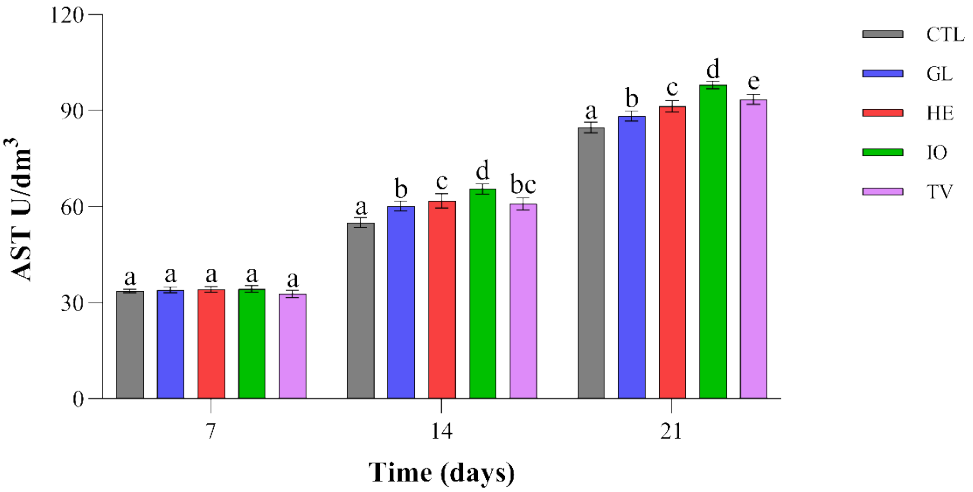

Figure 4. Aspartate transaminase (AST) activity in the haemolymph of Carniolan honey bees after 7, 14, and 21 days of feeding with fungal extracts (**GL:** *G. lucidum*, **HE:** *H. erinaceus*, **IO:** *I. obliquus*, and **TV:** *T. versicolor*, each at a concentration of 4%) and syrup alone (CTL: control). n = 20 bees per experimental group/time. Two-way ANOVA, interaction, F (8, 285) = 63.38, $p<0.0001$; time, F (2, 285) = 37353, $p<0.0001$; fungal extract, F (4, 285) = 234.0, $p<0.0001$. Statistically significant differences among treatments within each age group (7, 14, and 21 days) are indicated by letters. Data are presented as means ± standard deviation.

Activities of antioxidant enzymes

The activities of antioxidant enzymes (CAT, GPx, GST, and SOD), and TAC levels were measured in the bees' haemolymph after 7, 14, and 21 days of feeding with fungal extracts. As shown in Figures 5-9, the activities of all enzymes studied here increased with the bees' age.

CAT activity

The CAT activity in the haemolymph of bees from the GL, HE, IO, and TV groups was higher after 7, 14, and 21 days compared to the CTL group (Fig. 5), with a statistically significant difference ($p<0.0001$). The highest mean values across all analysed periods were observed in the IO group (7 days: 8.909, 14 days: 14.342, and 21 days: 19.038), while the lowest mean values were found in the CTL group (7 days: 6.648, 14 days: 8.706, and 21 days: 13.397).

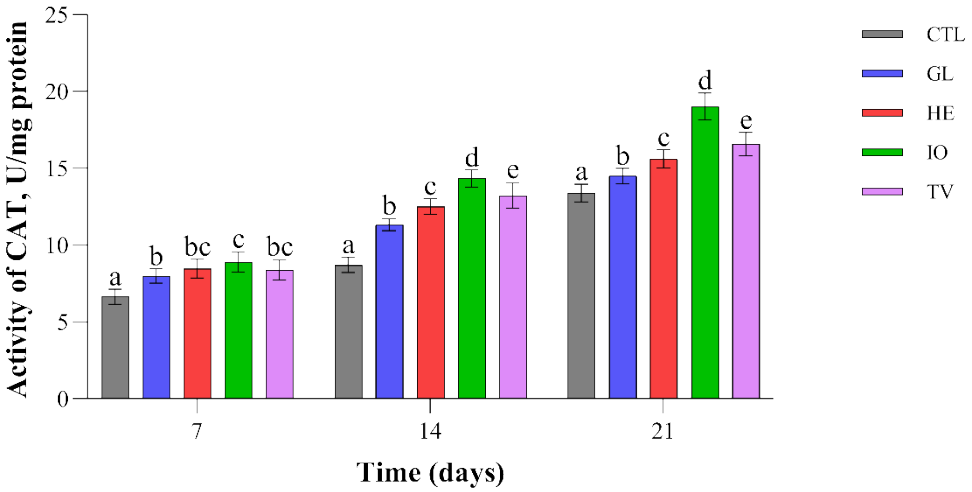

Figure 5. Catalase (CAT) activities in the haemolymph of Carniolan honey bees after 7, 14, and 21 days of feeding with fungal extracts (**GL:** *G. lucidum*, **HE:** *H. erinaceus*, **IO:** *I. obliquus*, and **TV:** *T. versicolor*, each at a concentration of 4%) and syrup alone (**CTL:** control). n = 20 bees per experimental group/time. Two-way ANOVA, interaction, $F(8, 285) = 40.95$, $p<0.0001$; time, $F(2, 285) = 4004$, $p<0.0001$; fungal extract, $F(4, 285) = 453.6$, $p<0.0001$. Statistically significant differences among treatments within each age group (7, 14, and 21 days) are indicated by letters. Data are presented as means ± standard deviation.

GPx activity

The GPx activity in the haemolymph of bees treated with fungal extracts did not differ statistically from the bees from the CTL group after 7 days ($p > 0.05$). However, the GPx activity was higher in the GL, HE, IO, and TV groups after 14 and 21 days ($p<0.0001$) compared to the CTL group (Fig. 6), which exhibited the lowest mean values (14 days: 25.597, and 21 days: 34.619). The bees from the IO group showed the highest mean values (14 days: 31.473, and 21 days: 42.158).

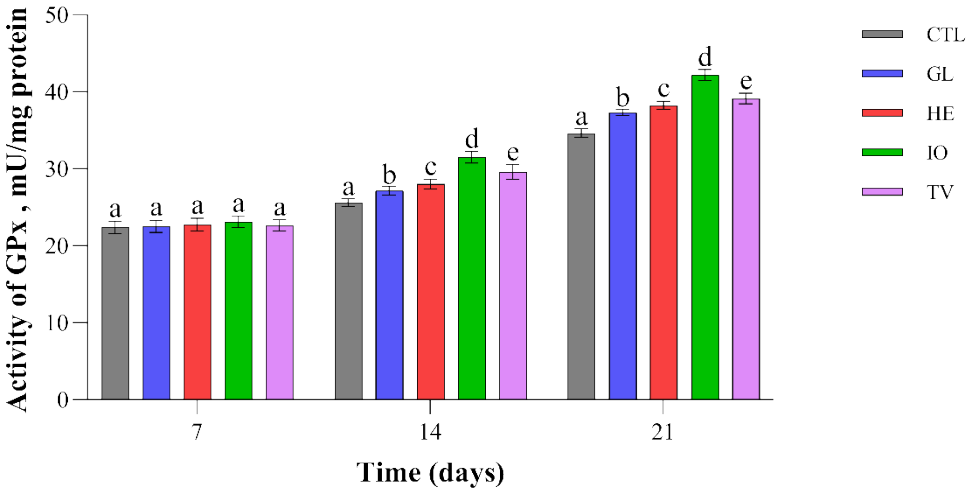

Figure 6. Glutathione Peroxidase (GPx) activity in the haemolymph of Carniolan honey bees after 7, 14, and 21 days of feeding with fungal extracts (**GL:** *G. lucidum*, **HE:** *H. erinaceus*, **IO:** *I. obliquus*, and **TV:** *T. versicolor*, each at a concentration of 4%) and syrup alone (**CTL:** control). n = 20 bees per experimental group/time. Two-way ANOVA, interaction, F (8, 285) = 74.49, p<0.0001; time, F (2, 285) = 13103, p<0.0001; fungal extract, F (4, 285) = 384.0, p<0.0001. Statistically significant differences among treatments within each age group (7, 14, and 21 days) are indicated by letters. Data are presented as means ± standard deviation.

GST activity

The GST activity in the haemolymph of bees from the GL (p = 0.0118), HE (p = 0.0006), IO (p<0.0001), and TV (p<0.0001) groups was significantly higher after 7 days compared to bees from the CTL group (Fig. 7). After 7 days, the highest mean value was observed in the TV group (10.199), while the lowest mean value was recorded in the CTL group (8.767). After 14 and 21 days, the increase in GST activity was even higher in all experimental groups when compared to the CTL group (p<0.0001), with the IO group exhibiting the highest mean values (14 days: 18.517, and 21 days: 22.372) and the CTL group the lowest (14 days: 13.390, and 21 days: 14.628).

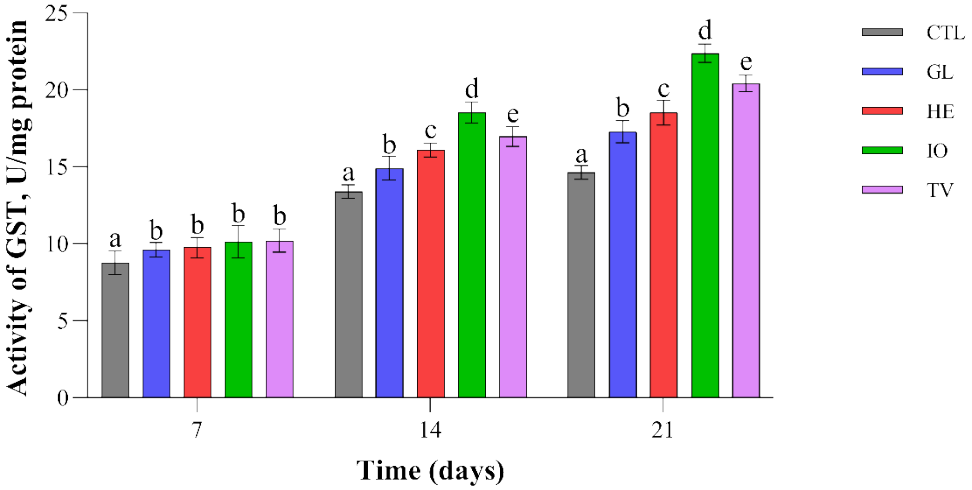

Figure 7. Glutathione s-transferase (GST) activity in the haemolymph of Carniolan honey bees after 7, 14, and 21 days of feeding with fungal extracts (**GL:** *G. lucidum*, **HE:** *H. erinaceus*, **IO:** *I. obliquus*, and **TV:** *T. versicolor*, each at a concentration of 4%) and syrup alone (**CTL:** control). n = 20 bees per experimental group/time. Two-way ANOVA, interaction, F (8, 285) = 66.60, p<0.0001; time, F (2, 285) = 4659, p<0.0001; fungal extract, F (4, 285) = 436.5, p<0.0001. Statistically significant differences among treatments within each age group (7, 14, and 21 days) are indicated by letters. Data are presented as means ± standard deviation.

SOD activity

After 7 days, the SOD activity in the haemolymph of bees from the GL, HE, IO, and TV groups was similar to that in the CTL group (Fig. 8), with no statistically significant difference observed (p>0.05). In contrast, after 14 and 21 days, all the experimental groups showed increased SOD activity compared to the CTL group (p<0.0001). The highest mean values were recorded in the IO group (14 days: 4.227, and 21 days: 9.498), whereas the lowest mean values were observed in the CTL group (14 days: 2.674, and 21 days: 5.311).

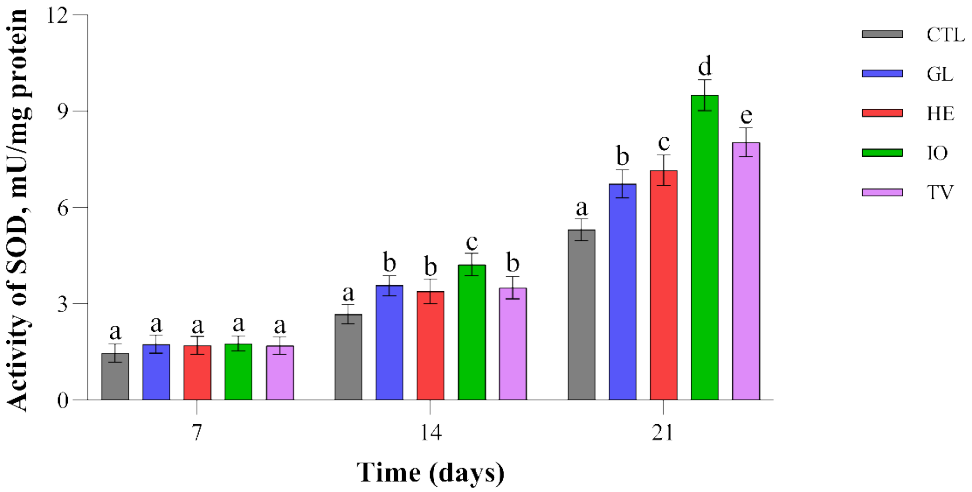

Figure 8. Superoxide dismutase (SOD) activity in the haemolymph of Carniolan honey bees after 7, 14, and 21 days of feeding with fungal extracts (**GL:** *G. lucidum*, **HE:** *H. erinaceus*, **IO:** *I. obliquus*, and **TV:** *T. versicolor*, each at a concentration of 4%) and syrup alone (**CTL:** control). n = 20 bees per experimental group/time. Two-way ANOVA, interaction, F (8, 285) = 90.02, p<0.0001; time, F (2, 285) = 6609, p<0.0001; fungal extract, F (4, 285) = 249.5, p<0.0001. Statistically significant differences among treatments within each age group (7, 14, and 21 days) are indicated by letters. Data are presented as means ± standard deviation.

Levels of TAC

TAC levels in the haemolymph of bees were higher in all fungal extract groups across all analysed periods compared to those from the CTL group (Fig. 9). After 7 days, the greatest difference compared to the CTL group was observed in the bees from the IO group (p<0.0001), followed by HE (p = 0.0032), TV (p = 0.0055), and GL (p = 0.0317). After 14 and 21 days, the GL, HE, IO, and TV groups showed the same statistically significant difference (p<0.0001) compared to the CTL group. As with the other parameters results already described, the highest mean values were recorded in the IO group (7 days: 48.691, 14 days: 57.897, and 21 days: 60.861), and the bees from the CTL group showed the lowest (7 days: 47.149, 14 days: 52.408, and 21 days: 55.554).

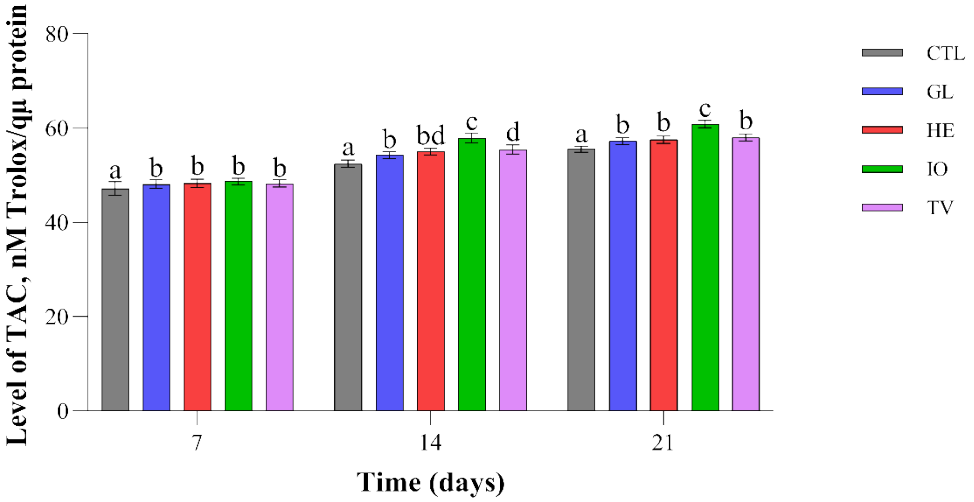

Figure 9. Total antioxidant capacity (TAC) levels in the haemolymph of Carniolan honey bees after 7, 14, and 21 days of feeding with fungal extracts (**GL:** *G. lucidum*, **HE:** *H. erinaceus*, **IO:** *I. obliquus*, and **TV:** *T. versicolor*, each at a concentration of 4%) and syrup alone (**CTL:** control). n = 20 bees per experimental group/time. Two-way ANOVA, interaction, F (8, 285) = 19.30, $p<0.0001$; time, F (2, 285) = 3374, $p<0.0001$; fungal extract, F (4, 285) = 176.0, $p<0.0001$. Statistically significant differences among treatments within each age group (7, 14, and 21 days) are indicated by letters. Data are presented as means ± standard deviation.

Discussion

Our results demonstrate that the consumption of fungal extracts can increase the survival rate of Carniolan honey bees, as well as the activities of metabolic markers (ALP, ALT, and AST), antioxidant enzymes (CAT, GPx, GST, and SOD) and TAC levels. The parameters chosen in the present study are commonly associated with honey bee health, especially physiological status and responses to metabolic and oxidative stress[46,30]. Moreover, supplementation with other natural compounds (e.g. cannabidiol) has also been shown to prolong bee lifespan and enhance proteolytic system and antioxidant activities[30], underscoring the relevance of using natural compounds, such as fungal extracts, to develop stronger and healthier honey bee colonies.

The results of the survival probability showed that *G. lucidum* at a concentration of 4% prolonged the lifespan of Carniolan honey bee foragers by 16.39% compared to the CTL group (Fig. 1). Comparable results were reported by Parish et al.[47], where honey bee workers exhibited

an increased lifespan after consuming diets containing fungal spores (*Botrytis cinerea*, *Cladosporium* sp., and *Colletotrichum acutatum*) combined with pollen. In addition, Stamets et al.[40] reported that extracts of polypore mushroom mycelia (*Fomes*, and *Ganoderma*) significantly decreased the levels of DWV and LSV in honey bees, demonstrating the health benefits of these extracts as well as their antimicrobial compounds. Furthermore, since forager bees face a wide variety of environmental threats while foraging, the present findings along with existing literature suggest that fungal extracts may serve as a beneficial supplement for honey bees.

Regarding the metabolic markers measured in this study (ALP, ALT, and AST), these enzymes play an important role in detoxification mechanisms and can provide useful information about the physiological status of honey bees[48]. As shown in the results (Figs. 2-4), all the fungal extract groups (*G. lucidum*, *H. erinaceus*, *I. obliquus*, and *T. versicolor*, each at a concentration of 4%) increased the levels of ALP, ALT, and AST after 14 and 21 days, with *I. obliquus* presenting the highest values, which suggests enhanced biochemical defence levels in honey bees over time. Similar results were found with other natural compounds, Skowronek et al.[46] observed higher activities of ALP, ALT, and AST over time in honey bees fed with cannabidiol in sugar syrup and on a cotton strip. Strachecka et al.[49] also reported elevated activities of enzymatic biomarkers (ALP, ALT, AST) in honey bee workers treated with curcumin, suggesting improved health and vitality of those honey bees. In contrast, decreased activities of the same enzymatic biomarkers were observed in honey bees after exposure to pesticides[48,50], highlighting that the results observed in this study indicate beneficial effects on honey bee health.

It is worth noting that the study involved bees from hives where frames remained in the same position throughout the experimental period. These bees likely had contact with the fungal extracts during their preimaginal development through royal jelly supplied to them by their nurse bees. Immune system stimulation during larval development by biostimulants such as phenylbutyrate- and phenylacetylglutaminate has

been previously documented[51-53]. The mechanisms underlying this stimulation by fungal extracts are unknown and therefore indicate future research directions. Particularly, future work should investigate whether fungal extracts: (1) directly stimulate the immune system of adult bees, (2) enhance the nutritional quality of royal jelly produced by nurse bees for the larvae, from which the workers emerge, or (3) pass into royal jelly and directly stimulate the immune systems of the larvae and subsequently the adult bees.

In line with the results for metabolic markers, the antioxidant enzymes studied (CAT, GPx, GST, and SOD) and TAC levels showed higher levels over time in all fungal extract groups, except for CAT, GST, and TAC after 7 days (Figs. 5-9). Antioxidant enzymes play an essential role in protecting cells against reactive oxygen species (ROS), which can be generated by various factors (e.g. environmental and physiological) and may induce oxidative damage to nucleic acids (DNA and RNA), proteins, and membrane lipids, thereby disrupting cellular homeostasis[54,55]. According to Ahmad[56], *G. lucidum* contains approximately 400 biologically active constituents, some of which possess antioxidant properties. Aligning with our findings, Cui et al.[57] reported a diversity of fungi associated with *A. m. ligustica*, pointing out that some of them have antibacterial and antioxidant activities.

It is proposed that higher activities of antioxidant enzymes have a positive effect on honey bee health, as evidenced by the prolonged lifespan of forager bees in the GL group. A similar pattern was observed in the IO group, which exhibited the highest antioxidant enzyme activities and no adverse effects on survival probability. Although the difference was not statistically significant, the lifespan in this group was 10.65% higher than that of bees in the CTL group. During the ageing process, increased ROS production in honey bees can potentially lead to oxidative stress, contributing to cellular damage and eventual cell death[54]. Therefore, protection from oxidative stress through an enhanced immune system is desired in order to achieve prolonged survival.

Given the observed influence of fungal extracts on survival, metabolic markers and antioxidant enzymes in honey bees in this study, it is important to point out that these extracts have also been previously investigated for their antimicrobial, antiviral, and anticancer properties in other organisms and under laboratory conditions[40, 58-61]. The medicinal benefits of *G. lucidum* for humans have been extensively studied and thoroughly documented over the years[62-65]. Similarly, according to Qiu et al.[61], *H. erinaceus* contains bioactive compounds with anticancer properties as well as antioxidant properties that regulate oxidative stress and are capable of enhancing the immune system in humans. Regarding *I. obliquus*, which showed the highest levels of the metabolic markers and antioxidant enzymes studied here, its capacity to scavenge free radicals and protect cells against oxidative stress has also been previously reported in human keratinocyte cells[66]. In addition, *I. obliquus* has been suggested to be potentially active against SARS-CoV-2[67]. Similarly, *T. versicolor* has also been associated with immune-boosting effects, digestive benefits, cardiovascular and anti-inflammatory effects[68]. However, despite their great potential, further studies are needed to fill the knowledge gap regarding the benefits of these fungal extracts on honey bees.

Since honey bees are facing many challenges in Europe[69] and all over the world[70], the use of natural compounds might be an alternative to promote healthier colonies and sustainable beekeeping.

The data presented here show that feeding Carniolan honey bees with fungal extracts can increase their lifespan, as well as the activities of metabolic markers and antioxidant enzymes. These findings indicate that fungal extracts may be used as a supplement to enhance the immune system function in honey bees, potentially contributing to colony fitness. Nevertheless, further studies should investigate the potential effects of these fungal extracts on cells and tissues and explore the effects of short- and long-term exposure.

Materials and Methods

Fungal extracts preparation

Fungal Strains

Axenic cultures of *G. lucidum*, *H. erinaceus*, *I. obliquus*, and *T. versicolor* were obtained from the fungal culture collection of MycoMedica Ltd., Slovenia. Cultures were transferred onto potato dextrose agar (PDA) and incubated for two weeks at 23 °C. When the PDA surface was overgrown, mycelia from a 12 cm diameter Petri dish were dispersed into 1 L of distilled sterile water using a Waring blender. 70 mL of the obtained liquid *inoculum* was used to inoculate 3 kg of the sterilised substrate.

Substrate media preparation

Millet grains were used as a growing substrate. Grains were cooked in boiling water, and excess water was drained off to reach a moisture level of 50%. Three kilograms (wet weight) were packed into a polypropylene bag with breathing filters and sterilised for 20 minutes at 121 °C and 1.15 bars of pressure. After inoculation, substrate bags were incubated for two months at 23 °C, 65% relative air humidity, and eight hours of light daily.

Preparation of fungal extracts

After incubation, 1 kg of each substrate overgrown with fungal biomass was broken into smaller pieces and blended into 2 L of 50% ethanol using an immersed kitchen blender. After five days of maceration, the material liquid was centrifuged at 4200 rpm, and the supernatant was stored at 3 °C until used in further bee experiments.

Experimental assay

Fifteen colonies of Carniolan honey bees from two apiaries were used in this study for laboratory and field assays. The first apiary, with ten colonies (of which five were used) of *A. m. carnica* housed in Alberti-Žnideršič (AŽ) hives, was located at the Botanical Garden of the University of Maribor (46°30'16.5"N, 15°38'01.2"E) in the municipality of Hoče, Slovenia. The second apiary, which housed 22 colonies (of which 10 were used) in AŽ hives, was located in “Lovrenc na Pohorju”, in the northern

part of the eastern Pohorje region, at an altitude of approximately 520 m above sea level (46°31'36.2"N, 15°22'39.5"E). The colonies were monitored prior to the study, and only the healthy ones were selected. The laboratory experiments were conducted at the Faculty of Agriculture and Life Sciences – University of Maribor (FKBV-UM), Slovenia.

Feeding in the laboratory to assess survival probability

Forager bees from five colonies were sampled from the apiary located at the Botanical Garden as they were flying back to their colonies and then transported to the FKBV - UM. The bees were kept in plastic cups (minimum diameter of 7.5 cm and height of 12.5 cm) with 80 aeration holes (3 mm diameter) covered by a plastic Petri dish (8.5 cm x 8.5 cm) with filter disc paper for improved hygiene (100 mm, 84 g/m2, grade 388), modified to serve as bioassay chambers or cages, and placed in an incubator at 31 ºC (± 1) with 70% (± 5) relative humidity in darkness. Four experimental treatments and an untreated control group of bees were established. Each experimental group consisted of 20 bees in five replicates (totalling 100 bees per group), and was divided as follows: *G. lucidum* (GL), *H. erinaceus* (HE), *I. obliquus* (IO), and *T. versicolor* (TV). Fungal extracts were offered in the form of a liquid diet (syrup) to the caged bees *ad libitum*, with the 4% concentration (v/v) based on preliminary studies conducted by our team. The control (CTL) consisted of untreated bees fed only sugar syrup (50% water + 50% sugar). Each day, the number of dead bees removed from the plastic cup was recorded, and the data was collected until all the bees had died. The methodology followed the guidelines outlined in the Organisation for Economic Co-operation and Development (OECD) Test No. 245 (OECD, 2017).

Feeding colonies with fungal extracts in the apiary

The colonies used in the field experiment (n = 10) were located at the apiary in “Lovrenc na Pohorju” and were fed from March to April 2023.

They were divided into the same treatments described above, with two colonies per treatment. Feeding was conducted for three consecutive weeks on Mondays, Wednesdays, and Fridays at approximately 6:00 PM. In the fungal extract treatments (GL, HE, IO, and TV), each colony was offered 333 mL of syrup containing 4% of the respective fungal extract. The CTL group received the same amount of syrup, composed of 50% water and 50% sugar, without the addition of fungal extracts. Each colony received a total of 3 L of food. No Varroa mites were found on the bottom boards, nor were *Nosema* spores detected in dissected bees from the colonies during the period in which the experiment was performed.

Sampling bees in the apiary

Bees were sampled for subsequent analysis on days 7, 14, and 21, starting from the beginning of the feeding period. On these days, 30 bees were collected from each colony, specifically from the honey storage frames, after they had ingested the syrup containing the fungal extracts. The bees were then placed in disposable plastic cups for transportation to FKBV - UM, where the haemolymph was collected. In this study, a total of 900 bees were sampled from the apiary.

Haemolymph collection

Fresh haemolymph was collected from 10 bees per colony (n = 20 bees/treatment/time) in each treatment group at 7, 14, and 21 days following fungal extract feeding. Collection was performed using a micropipette from the thoracic region, as described by Domingues et al.[71] and Balsamo et al.[72]. The volume of haemolymph from each bee (3 µL) was added individually to a sterile microtube containing 197 µL of 0.6% NaCl and then promptly chilled at -25°C for future biochemical analyses. The collected haemolymph (n = 300 samples) was sent to the University of Life Sciences in Lublin, Poland, where the biochemical analyses were conducted.

Biochemical analysis

Metabolic markers

Activities of alkaline phosphatase (ALP), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined using monotests from Cormay (Lublin, Poland) according to the manufacturer's procedure.

Antioxidant activities

The following parameters were determined in haemolymph solutions:

- Catalase (CAT) activity according to the method described in the commercial kit Catalase Assay Kit, Cayman Chemical Company, East Ellsworth Road, Ann Arbor, USA, Item: 707002;
- Glutathione peroxidase (GPx) activity according to the method described in the commercial kit Glutathione Peroxidase Assay Kit, Sigma Aldrich, Schnelldorf, Germany, no. MAK437-1KT;
- Glutathione S-transferase (GST) activity according to the method described in the commercial kit Glutathione S-transferase Assay Kit, Sigma Aldrich, Schnelldorf, Germany, no. MAK 435-1KT;
- Superoxide dismutase (SOD) activity according to the method described in the commercial kit SOD Assay Kit, Sigma Aldrich, Schnelldorf, Germany, no. 19160-1KT-F;
- Total antioxidant capacity (TAC) according to the method described in the commercial kit Antioxidant Assay Kit, Cayman Chemical Company, East Ellsworth Road, Ann Arbor, USA, Item: 709001.

The antioxidant enzyme activities were calculated per 1 mg of protein.

Statistical analysis

The data analysis was carried out in the GraphPad Prism version 10.5.0 (774) software (GraphPad Prism Software, Inc., San Diego, CA,

USA). Survival probability was determined by Kaplan-Meier survival analysis, followed by multiple comparisons of groups with the Holm-Sidak method. The activities of metabolic markers (ALP, AST, and ALT), and antioxidant enzymes (GPx, SOD, and TAC) were compared using a mixed-model two-way ANOVA, followed by Tukey's multiple comparisons test. Significance level was set at $p<0.05$, and the graphs display mean ± standard deviation.

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Author contributions

L.S.A., V.V., C.E.C.D., and A.Gc. designed the work; L.S.A., V.V., and C.E.C.D. performed experiments; L.S.A., V.V., P.S., A.S., M.G., A.G., and C.E.C. acquired data; L.S.A., and C.E.C.D. interpreted data; L.S.A., and V.V. wrote the main manuscript; A.Gc. coordinated the project. All the authors substantively revised the draft of the manuscript. All authors read and approved the final version of the manuscript.

Corresponding author

Correspondence to Leticia S. Ansaloni.

Competing interest

The authors declare no competing interest.

Data availability

All requests for data or datasets should be addressed to Leticia S. Ansaloni.