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# Fine-scale assessment of Chlorella syrup as a nutritional supplement for honey bee colonies

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Habitat loss, climate change, and global agriculture have a non-negligible effect on the reduced abundance and diversity of floral resources. Malnutrition and nutritional stress are consequences of the combination of these factors with considerable impact on honey bee health and colony losses. The solution to inadequate natural sources for beekeeping is the additional feeding of honey bee colonies with food supplements. The algae *Chlorella* is a natural food source, with a nutrient profile similar to natural pollen, thus it has promising application in beekeeping. We evaluated *Chlorella vulgaris* syrup as a dietary supplement in the view of the oxidative stress that may be caused by long term administration to the colonies. Consuming *Chlorella* syrup did not influence the activity of digestive enzymes of summer honey bee workers, however, lipase activity insignificantly increased. After *Chlorella* application to colonies, we also observed insignificantly higher gene expression of antioxidant enzymes *catalase* and *superoxid dismutase1* in adult workers; however, in larvae the expression of those genes was not affected. Surprisingly, the gene expression did not correspond with enzyme activity in adult bee abdomens. In *Chlorella* fed colonies, we recorded a higher concentration of vitellogenin, which plays multiple roles in honey bee physiology, i.e., antioxidant, storage protein, or immunity-related functions. Our new findings brought evidence that *Chlorella* did not negatively affect the digestion or oxidative balance of honey bees, thus its application as a pollen supplement can be fully recommended for maintaining the health of honey bee colonies during periods of dearth.

## KEYWORDS

pollen supplement, microalgae, oxidative stress, beekeeping, malnutrition

## Highlights

- *Chlorella* syrup was well accepted by bees and larvae
- The digestive system of bees is not influenced by consuming *Chlorella* syrup
- *Chlorella* syrup consumption increases gene expression of antioxidant enzymes without affecting their activity
- *Chlorella* consumption stimulated vitellogenin production

## Introduction

Over the past few decades, increased honey bee colony losses have been reported worldwide (Kulhanek et al., 2017; Gray et al., 2019), which brings the health and nutrition of honey bees to the forefront of general interest (Brodschneider and Crailsheim, 2010; Meixner and Le Conte, 2016). Habitat loss, climate change, and global agriculture have a non-negligible effect on the reduced abundance and diversity of floral resources. Increasing areas of non-forage habitats or monocultures have led to a strong emphasis on the quality of food sources and their impact on the vitality of bees as well (Smart et al., 2016; Alaux et al., 2017; Smart et al., 2019). Diversity and availability of nutritional resources, especially pollen, are known to be essential for the proper functioning of the honey bee colony and the immune system of individual bees (Alaux et al., 2010a; Scofield and Mattila, 2015; Di Pasquale et al., 2016; Frias et al., 2016; Danihlik et al., 2018). Therefore, malnutrition and nutritional stress can make a remarkable contribution to high colony losses (Potts et al., 2010; Goulson et al., 2015; Branchiccela et al., 2019). The solution to inadequate natural sources for beekeeping during periods of dearth is the additional feeding of honey bee colonies with commercial protein supplements. The use of commercial supplements is currently the subject of intense research due to the need to holistically determine the effectiveness of protein supplementation using diets that consider both individual bee and colony-level health metrics (Brodschneider and Crailsheim, 2010; Danihlik et al., 2018; Wright et al., 2018).

Microalgae have been tested as a potential food supplement for honey bees (Eremia et al., 2013; Jehlík et al., 2019; Ricigliano, 2020; Ricigliano and Simone-Finstrom, 2020; Jang et al., 2022; Ricigliano et al., 2022). The nutritional quality of algae is similar to that of pollen, with a high content of proteins, nutritionally relevant lipids, essential amino acids and micronutrients such as vitamins and minerals, especially within two genera—*Arthrospira* (also known as spirulina) and *Chlorella* (Ricigliano, 2020). Protein supplementation using *Chlorella vulgaris* was reported to increase honey bee colony productivity, as noted by increased brood production and honey stores (Eremia et al., 2013). Additionally, it has been described that organic extracts from microalgae have antimicrobial activity. Hence, the microalgae-based feed could have additional prophylactic properties for honey bee colonies (Catarina Guedes et al., 2011; Salem et al., 2014; Dostálková et al., 2021).

Little is known regarding how dietary supplements affect individual bees at the level of physiological and molecular parameters. Effectiveness of the immune system, modifications of biochemical and physiological reactions or capacity of anti-oxidative defence reactions should be studied to more holistically assess any additional impacts (positive or negative) of dietary supplements, particularly those during different developmental and behavioural states of the bee. Such fine-scale measures are often neglected but can provide a more complete picture of the value of potential nutritional products redundant (Ricigliano et al., 2022). Recent studies indicate, that supplementing bees with pollen can improve honey bee health and positively influence stimulation of the immune

system on both colony and individual levels (Alaux et al., 2010b; Di Pasquale et al., 2013; Annoscia et al., 2017; Danihlik et al., 2018; Degrandi-Hoffman et al., 2020). Recent work on spirulina found that in caged bees, a diet of natural pollen or spirulina resulted in comparable proteome expression patterns indicating that microalgal supplements may be a sustainable and effective diet matching honey bee needs (Ricigliano et al., 2021). In a previous study, we documented the potential for *Chlorella* as a dietary supplement for honey bees (Jehlík et al., 2019). We found that colonies fed *Chlorella* had positive effects of supplementation during the spring on both colony level (i.e., increased brood production) and individual level metrics (i.e., larger hypopharyngeal glands in nurse bees). Pre-winter colony supplementation provided initial evidence of the modulatory influence of *Chlorella* feeding on basic digestive enzymes, which was supported by cage studies that also determined that the algal diet influenced transcript levels of endocrine markers involved in cell signal pathways (Jehlík et al., 2019).

As microalgae are rich in antioxidants (phenolics, carotenoids, and other photosynthetic pigments) (Li et al., 2007; Goiris et al., 2012), within this study we also aimed to investigate *Chlorella*'s antioxidant properties when consumed by bees. Antioxidants can scavenge and detoxify by-products of aerobic metabolism—free radicals and reactive molecules termed as reactive oxygen species (ROS). In insect tissues, ROS are constantly produced by a diverse array of metabolic processes, including mitochondrial respiration, multiple oxidases enzymes, or xenobiotic degradation. Increased ROS levels are caused by the imbalance between the ROS production rate and the antioxidant capacity of the organism. Enhanced levels of ROS are harmful as they can lead to oxidative damage of important biomolecules such as proteins, lipids, and nucleic acids, and thus negatively affect basic biochemical and physiological processes (Sies et al., 2017). Primary protection against ROS accumulation and oxidative stress is ensured by key antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT) along with glutathione- and thioredoxin-dependent peroxidases (Weirich et al., 2002; Corona and Robinson, 2006). SOD primarily converts superoxide radical anions ( $O_2^-$ ) generated by aerobic metabolism to hydrogen peroxide and oxygen. There are two SOD isoforms in all eukaryotes, cytosolic SOD1 (copper; zinc-containing SOD) and mitochondrial SOD2 [manganese-containing SOD (Weirich et al., 2002)]. CAT is an enzyme primarily located in the peroxisomes and driving the decomposition of hydrogen peroxide to water and oxygen (Ahmad et al., 1988; Ahmad and Pardini, 1990).

Vitellogenin (Vg) is known to play crucial roles in bee reproduction, nutrition and immunity (Salmela and Sundström, 2018) as well as longevity of honey bees (Fluri et al., 1982; Amdam et al., 2005). Vg synthesized in the fat body has been suggested to fulfill also an important function in the antioxidant system of honey bees. There is evidence that Vg might be preferentially oxidized, and thus represent an important component of antioxidant repertoire in bee hemolymph (Seehuus et al., 2006; Zhang et al., 2017).

This study aimed to conduct a fine-scale assessment of *Chlorella vulgaris* as a dietary supplement for honey bee colonies to more

holistically evaluate the long-term effects of the algal supplementation on individual bees. The effects of *Chlorella* were measured based on the activity of digestive enzymes, antioxidant enzyme activity and gene expression, and Vg production and gene expression. The results support the assumption that *Chlorella* can be used as a safe protein supplement for honey bees in support of bee homeostasis.

## Materials and methods

### *In vivo* *Chlorella* application to honey bee colonies

Honey bee colonies (*Apis mellifera carnica*) were kept in one apiary close to Krhová (49.4842989N, 17.9937911E) located in the Czech Republic. The colonies were at the strength of 11 occupied frames (42.5 × 17 cm) in one box (Langstroth style). Three control colonies (Control) were fed with APIVITAL® sugar syrup (Dobruška, Czech Republic). Three experimental colonies (Algae) were fed with 10% (w/v) *Chlorella* powder in APIVITAL® sugar syrup. *Chlorella* powder consisted of autotrophic alga *Chlorella vulgaris* provided by the Centre Algatech, Institute of Microbiology CAS, Třeboň, Czech Republic. Both groups, Control, and experimental (Algae) consumed a total of 3 liters of feed per colony over 2 weeks, equally 300 g of dry *Chlorella* powder for the algae treatment. After 2 weeks of feeding, approximately 300 adult bee workers and one frame with uncapped brood were collected from each hive. All samples were immediately frozen and stored at –80°C.

### Quantification of vitellogenin

For Vg quantification in larval and worker hemolymph, a common direct ELISA was used. The hemolymph was taken from the bee abdomens that were gently punctured with a pin, and the leaking hemolymph was collected by a capillary; one  $\mu\text{l}$  ( $n = 20$ ) was then used for Vg quantification. The primary anti-Vg antiserum (Koubová et al., 2021) was diluted 1:20,000 and secondary antibody – swine anti-rabbit IgG labelled with horseradish peroxidase (SwAR/HPR – LabNed) – was diluted 1:2,000. The reaction was visualized using 3,3',5,5'-tetramethylbenzidine (Sigma Aldrich, United States), and the absorbance of the product was determined at 450 nm in a microplate reader. The results were expressed as the absorbance normalized per 1  $\mu\text{l}$  of hemolymph.

### The activity of digestive enzymes in the adult worker midgut

The activities of basic digestive enzymes—proteases, amylases, and lipases—were determined in the midgut of the adult workers. The organs were dissected, weighed, homogenized by sonication in 0.2 M Tris pH 7.8, and centrifuged. The aliquots—0.1 midgut equivalents for proteases, 0.025 midgut equivalents for amylases, and 0.25 midgut equivalents for lipases—were tested for enzymatic activity.

### Protease assay

For the determination of protease activity the resorufin-casein kit (Roussel et al., 2015) was used according to the manufacturer's instructions. Sample extracts in 0.2 M Tris pH 7.8 were mixed with 0.4% resorufin-casein substrate and 0.02 M calcium chloride solution in microcentrifuge tubes. Afterward, the mixture was heated for 1 h at 37°C under gentle shaking. The reaction was terminated by 5% trichloroacetic acid, and the mixture was incubated for the next 10 min at 37°C. Non-hydrolyzed casein was then removed by centrifugation. The absorbance of the supernatant was measured at 490 nm. Protease activity was expressed in units of proteolytic activity per mg of fresh midgut weight. The activity unit (U) was defined as the amount of enzyme (mg) which caused an increase in optical density by 0.1 per min in 1 ml of the reaction mixture (Elpidina et al., 2001).

### Amylase assay

The 3,5-dinitrosalicylic acid (DNS) reagent was used for the determination of amylase activity as described previously (Kodrik et al., 2012). In brief, sample extracts were mixed with an equal volume of 2% starch (Sigma Aldrich, United States) in a phosphate buffer. The mixture was agitated for 40 min at 30°C, and the reaction was terminated by adding 200  $\mu\text{l}$  of 0.005% DNS solution. Subsequently, the solution was boiled at 100°C for 5 min, cooled, centrifuged (10,000 g 10 min), and the absorbance of the supernatant was determined at 550 nm. Amylase activity was expressed in  $\mu\text{mol}$  maltose (maltose was served as standard) per mg of midgut fresh weight (Bernfeld, 1955).

### Lipase assay

For determination of lipase activity, 4-methylumbelliferyl butyrate (4-MUB) was used (Roberts, 1985; Kodrik et al., 2012). In brief, the 5  $\mu\text{l}$  2 mM 4-MUB substrate diluted in dimethylsulfoxide (DMSO) was mixed with the 5  $\mu\text{l}$  sample extracts (diluted in 0.1 M acetic buffer, pH 5) and 195  $\mu\text{l}$  0.1 M acetate buffer, pH 5. The release of the fluorescent 4-methylumbelliferone (4-MU) was monitored at 5 min intervals for 30 min at 327 nm excitation and 449 nm emission with a Synergy 4 multi-mode microplate reader (BioTek Instr., Winooski, Vermont, United States). Lipase activity was expressed in nmol of 4-MU per min per mg of midgut fresh weight.

### Protein concentration and activity of catalase (CAT) and superoxide dismutase (SOD) in abdomens of adult worker bees

*Sample processing and concentration of proteins* - Bees were separated into body parts and the digestive system was removed from each bee. For further analysis, only abdomens were used. Samples were homogenized using TissueLyser II (Qiagen, Germany) using glass beads (3 mm in diameter, Roth, Germany). Then 200  $\mu\text{l}$  of 0.1 M potassium phosphate buffer pH 7.0 was added to the homogenate. Samples were centrifuged for 10 min at 12,000 g

and 4°C. The supernatant was used for further measurements. For all samples, protein concentration was determined according to Bradford (1976) by using Coomassie® brilliant blue G-250 (Sigma Aldrich, United States) and measured at 595 nm. Bovine serum albumin (Merck, Germany) was used as a calibration standard. Samples were measured in triplicates for both protein concentration and enzyme activities (i.e., CAT and SOD).

**Catalase activity** - for determination of CAT activity, extracts of bee abdomens were diluted 1:2 in potassium phosphate buffer pH 7.0. The enzyme reaction was measured according to Aebi (1984) on a Synergy H1 Multi-Mode Reader (BioTek Instr., Winooski, Vermont, United States). For the reaction, 220 µl of buffer and 10 µl of the sample were used; the reaction was initiated with 20 µl 150 mM H<sub>2</sub>O<sub>2</sub> (Lach-Ner, Czech Republic). Absorbance changes at 240 nm were recorded for 5 min at 30°C. CAT activity was expressed as the amount of decomposed H<sub>2</sub>O<sub>2</sub> (molar extinction coefficient  $\epsilon_{240} = 39,400 \text{ M}^{-1} \cdot \text{cm}^{-1}$ ) in 1 ml of bee extract.

**Superoxide dismutase activity** - abdomen extracts used for the measurements of SOD activity were diluted 50× in 0.02 M Tris buffer pH 8.2 containing 1 mM EDTA and then used for determination of SOD activity according to Marklund and Marklund (1974). A volume of 220 µl of Tris buffer and 20 µl of diluted sample was used for the reaction. For the initiation of the reaction 20 µl of 2 mM pyrogallol was pipetted into the well. The change in absorbance was measured for 5 min at 25°C with a wavelength of 420 nm. SOD activity was expressed in enzyme units (U) per one ml of bee extract. The unit is defined as the amount of enzyme which inhibits the auto-oxidation of pyrogallol by 50% (Marklund and Marklund, 1974).

## Quantification of relative gene expression in adult worker bee and bee larvae

To quantify relative gene expression, a pooled sample of 2 adult bees was used for RNA extraction. The bees were placed in plastic mesh bags (Bioreba, Switzerland), 1.2 ml of GITC lysis buffer containing 1% β-mercaptoethanol (Sigma Aldrich, United States) were added and a sample was homogenized using a pestle. For larvae samples, one larva was homogenized in homogenizer TissueLyser II (Qiagen, Germany). After homogenization 300 µl of GITC lysis buffer was added.

Total RNA was extracted from homogenates in GITC buffer using 300 µl of homogenate for adult bees and larvae samples. The NucleoSpin RNA Plus kit was used for RNA extraction according to the manufacturer's instructions (Marchery-Nagel, Germany). RNA was eluted in 60 µl of RNase-free water, then the concentration and quality of RNA samples were quantified by Synergy HT microplate reader (BioTek, Germany). RNA integrity was confirmed by electrophoresis in 1.1% (w/v) agarose gel stained by GelRed (Biotium, Fremont, CA, United States). Reverse transcription was performed with the Transcriptor High Fidelity kit (Roche, Switzerland) in 20 µl reaction volumes according to the

manufacturer's protocol with a final concentration of RNA 50 ng/µl per reaction. Relative gene expression was determined using SYBR Green (SYBR Select Master Mix, Applied Biosystems, United States) on CFX96 Touch Real-Time PCR Detection System (Bio-Rad, United States). Product quality and contamination were tested by measuring the melting curves of PCR products (60°C – 95°C, using increments of 0.5°C for 0.1 min). The relative expression of genes of interest (*catalase [CAT]*, *superoxide dismutase1 [SOD1]*, *superoxide dismutase2 [SOD2]*, *vitellogenin [Vg]*) to housekeeping genes (HKG) (*RPS5*, *EF-alpha*) was calculated according to Pfaffl (2001). The stability of housekeeping genes were tested by the Excel-based tool BestKeeper™ (Pfaffl et al., 2004). For primer sequences and stability of housekeeping genes see [Supplementary material S1](#).

## Statistical analysis

Statistica version 13 software (TIBCO Inc., Palo Alto, CA, United States) was used for the data analysis; OriginPro 2019b (64-bit) was used for creation the plots. Data were tested for normality by the Shapiro–Wilk test. Based on data distribution, it was decided whether to use non-parametric Mann–Whitney U test (Figures 1, 2, 3, 4A) or parametric t-test (Figures 4B, 5, 6).

Due to multiple testing, we used Bonferroni correction, with the level of significance set to  $\alpha = 0.004$ . The *p*-values ( $p < 0.05$ ) were added into graphs for informative reasons. The statistical power was calculated for each statistical test, for details see [Supplementary Table S2](#). The number of biological replicates is noted in the individual graphs; the colonies are represented equally in each group.

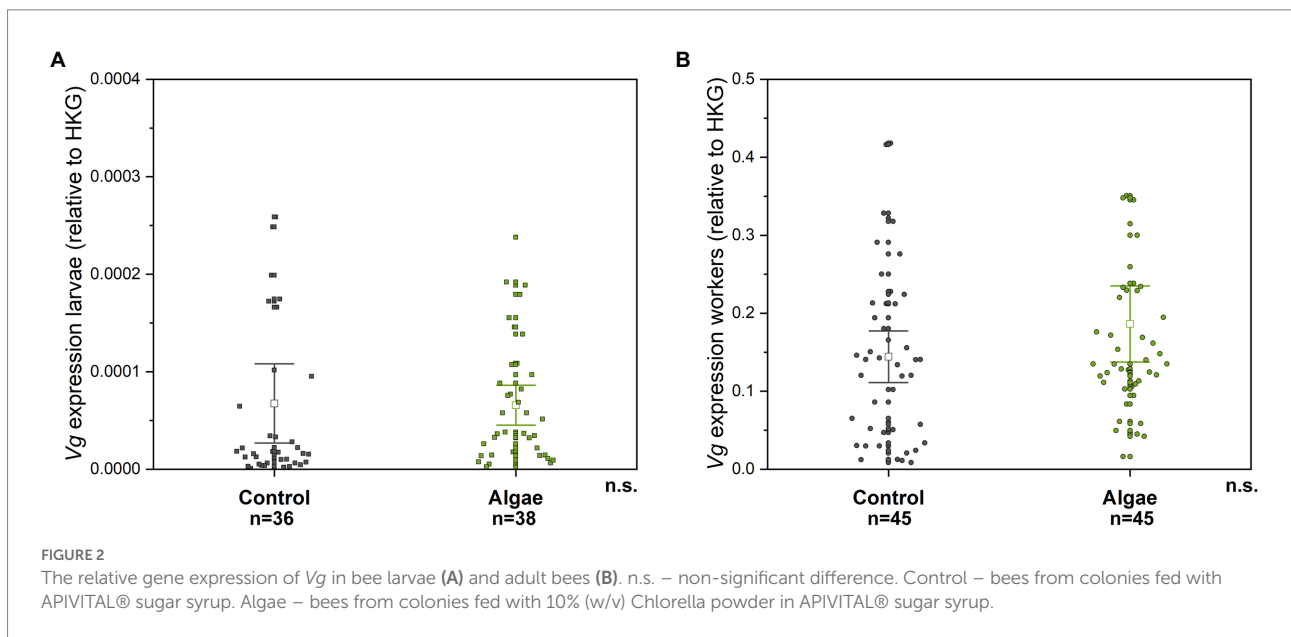
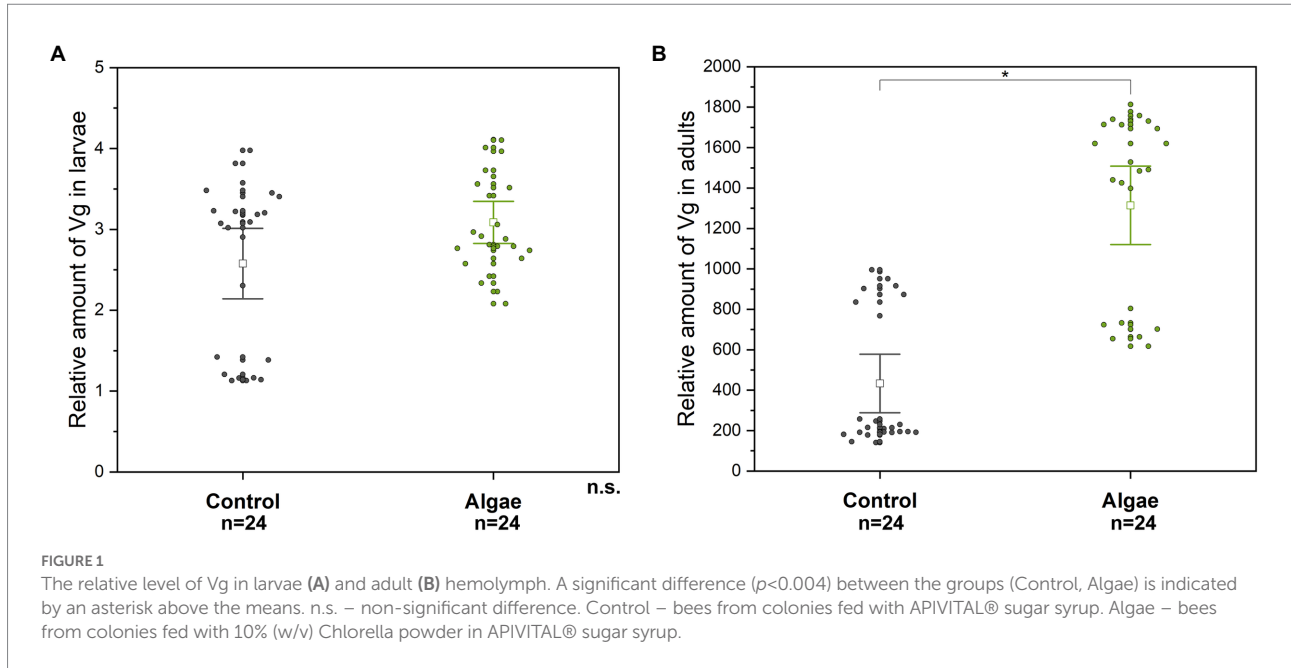
## Results

### Feeding Chlorella to honey bee colonies

During the feeding experiment, it was observed that bees collected the Chlorella-enriched syrup and stored it similarly to honey (Figure 7A). Notably, adult bee workers directly transferred Chlorella to larvae as part of their diet, which could be inferred by the green colour of the larval midgut (Figure 7B). The uptake of Chlorella syrup was also clearly visible by the green colour of dissected honey crops (Figure 7C).

### The effect of Chlorella on the worker bee digestive system

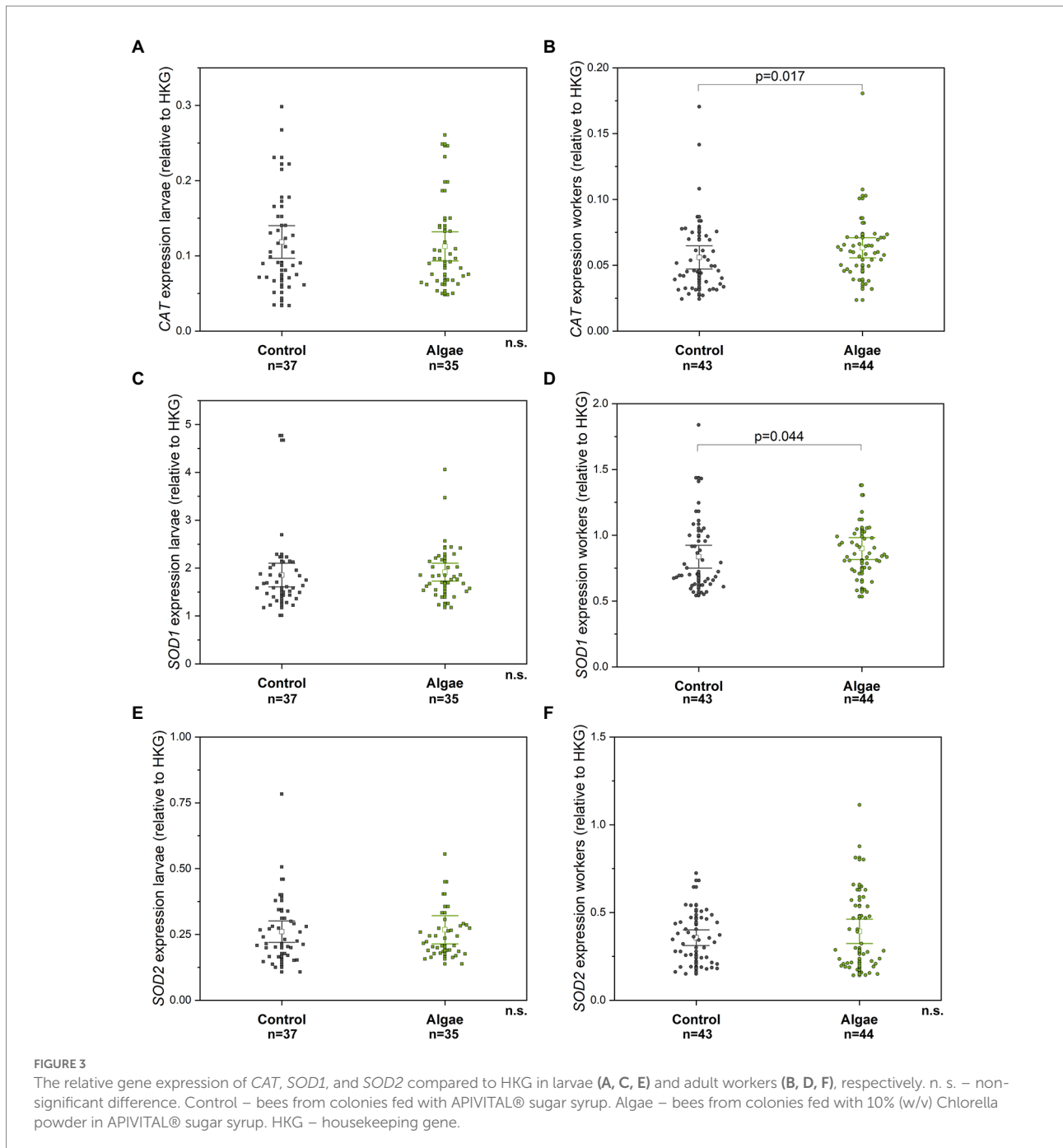
To determine the effect of the algal supplement on the bee digestive system, the weight of honey bee midgut and activity of digestive enzymes were measured in bees from colonies either fed Chlorella syrup or a control diet. After the two-week feeding



period, no effect was observed on the weight of the midgut mass of bees from Chlorella-fed colonies as compared to control bees ( $t$ -test,  $p = 0.188$ ,  $df = 52$ ) (Figure 5A). Similarly, activities of amylase and protease enzymes were not influenced by the consumption of Chlorella-enriched syrup (amylase,  $t$ -test,  $p = 0.221$ ,  $df = 36$ ; protease,  $p = 0.230$ ,  $df = 37$ ) (Figures 5B,C). Interestingly, the activity of lipase in worker midgut was higher in bees from control colonies (about 1.5-fold) than that in bees from colonies fed with Chlorella ( $t$ -test,  $p = 0.027$ ,  $df = 33$ ) (Figure 5D), however the difference was not statistically significant.

## Protein levels in honey bee extracts increased after Chlorella consumption

Extracts prepared from the abdomens of adult bees from Chlorella-fed colonies showed higher protein concentration ( $t$ -test,  $p = 0.013$ ,  $df = 82$ ) than the control group (Figure 6). The average concentration of proteins in extracts from abdomens was  $1140.8 \pm 723.4 \mu\text{g/ml}$  for bees from the Control group and  $1548.1 \pm 742.9 \mu\text{g/ml}$  for the Chlorella-fed group which indicates about a 1.4-fold insignificant increase.



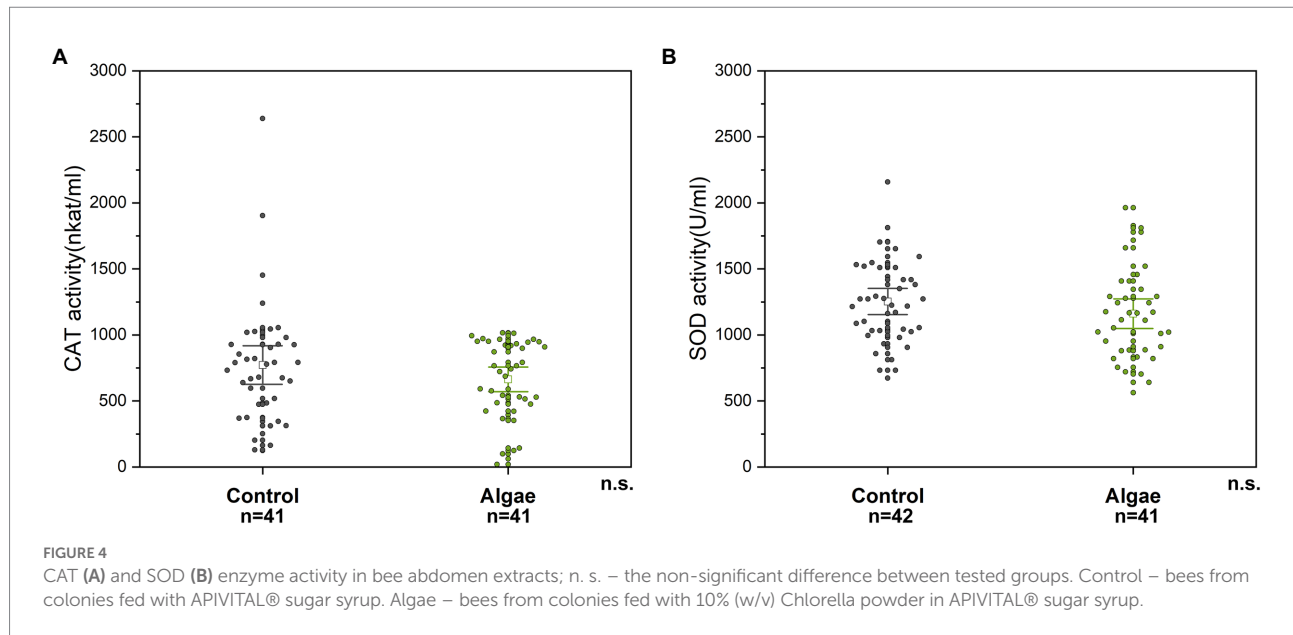
## The level of vitellogenin increases in adult bees after Chlorella consumption

The Chlorella supplemented diet did not influence the Vg level in the hemolymph of honey bee larvae (Mann–Whitney *U*-test,  $p=0.317$ ,  $Z=-1$ ) (Figure 1A). On the other hand, the relative level of Vg in the adult hemolymph was significantly higher (about 3 times) in the group fed with Chlorella (Mann–Whitney *U*-test,  $p=4 \times 10^{-6}$ ,  $Z=-4.629$ ) (Figure 1B) than in

controls fed with sugar solution only. The Vg level in larvae was several 100 times lower than that in adults.

## Vitellogenin gene expression was not influenced by Chlorella in adult workers or larvae

The relative gene expression of *Vg* gene transcripts was not significantly different between the control group and the



Chlorella-fed group in adult bees (Mann–Whitney U test,  $p=0.178$ ,  $Z=-1.348$ ) and in larvae (Mann–Whitney U test,  $p=0.0577$ ,  $Z=-1.898$ ), Figure 2. Similarly to Vg protein abundance, the expression of the Vg gene is around three magnitudes lower compared to adults.

### Chlorella consumption triggers gene expression of antioxidant enzymes (CAT, SOD1) In adult workers

The relative expression of genes for antioxidant enzymes (CAT, SOD1, SOD2) was measured in larvae and adult bees. No significant differences between the control and the group of the experimental larvae from Chlorella-fed colonies were observed for all genes tested (Mann–Whitney U test, CAT  $p=0.679$ ,  $Z=0.414$ ; SOD1  $p=0.137$ ,  $Z=-1.484$ ; SOD2  $p=0.952$ ,  $Z=0.061$ ) (Figures 3A,C,E). However, adult workers from colonies fed with Chlorella syrup showed insignificantly higher relative gene expression of CAT (Mann–Whitney U-test,  $p=0.017$ ,  $Z=-2.390$ ) (Figure 3B) and SOD1 (Mann–Whitney U-test,  $p=0.044$ ,  $Z=-2.016$ ) (Figure 3D) in comparison to control bees. SOD2 gene expression showed no differences between the two experimental groups (Mann–Whitney U-test,  $p=0.802$ ,  $Z=-0.250$ ) (Figure 3F).

### Activities of antioxidant enzymes are not influenced by Chlorella feeding

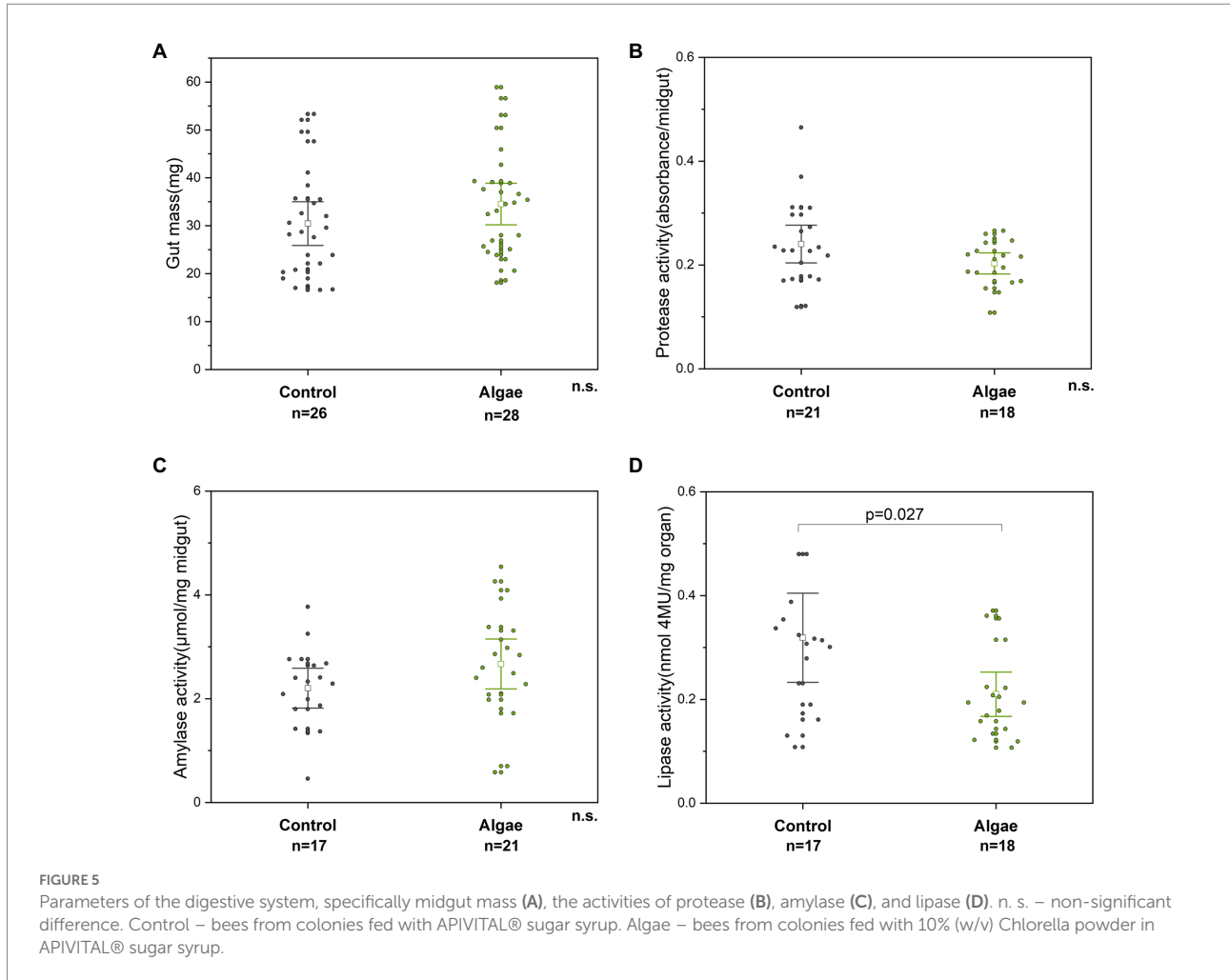
The activity of antioxidant enzymes CAT and SOD were determined in abdomens of honey bee workers with removed midgut. No differences in activity of CAT and SOD were detected

within two experimental groups tested (Mann–Whitney U test,  $p=0.450$ ,  $Z=0.747$  for CAT in abdomens; and  $t$ -test,  $p=0.219$ ,  $df=81$  for SOD in abdomens) (Figure 4).

## Discussion

The alga Chlorella was previously described as a promising nutritional source for honey bees (Ricigliano, 2020) with a positive effect on their development and physiology (Eremia et al., 2013; Jehlík et al., 2019). As the potential for using algae as nutrient supplementation for honey bees and other livestock is of increasing interest (Madeira et al., 2017; Wells et al., 2017; Nethravathy et al., 2019), we aimed to perform a fine-scale assessment of metabolic modulations, including antioxidant enzymes, digestive enzymes, and Vg levels. Our results complement previous studies on the colony and individual levels (Al-Saif et al., 2014; Jehlík et al., 2019) to provide a more holistic evaluation of the suitability of Chlorella supplementation for bee health and vitality. The feeding trial was performed in August 2019, which is at the end of beekeeping season when pollen, nectar, or honeydew sources are very limited in this region, thus effects caused by Chlorella supplementation should not be influenced by other nutritional resources. Importantly, this is also a relevant time for supplementation from a colony management perspective, as during this period of dearth, beekeepers feed proteinaceous supplements with expectations to improve bee health prior to overwintering (Mattila and Otis, 2007).

Honey bee colonies accepted the alga syrup well with a positive effect on their development, as previously reported for Chlorella by Eremia et al. (2013) and Jehlík et al. (2019) as well as *Arthrospira platensis* (spirulina) (Ricigliano and Simone-Finstrom, 2020). In our experiment, the syrup was



stored in honeycombs, which was clearly visible because of the typical green colour of the syrup. Consumption of algae was confirmed with the colour difference of larvae gut and honey bee crop. [Ehrenberg et al. \(2019\)](#) used synthetic compounds, such as Coomassie Brilliant Blue, as colourful dyes suitable for *in vitro* assays of food intake in honey bee larvae. We hypothesize, that Chlorella in moderate concentrations could be used as an effective, low-cost and environment-friendly dye, e.g., in feed intake and in hive storage related studies.

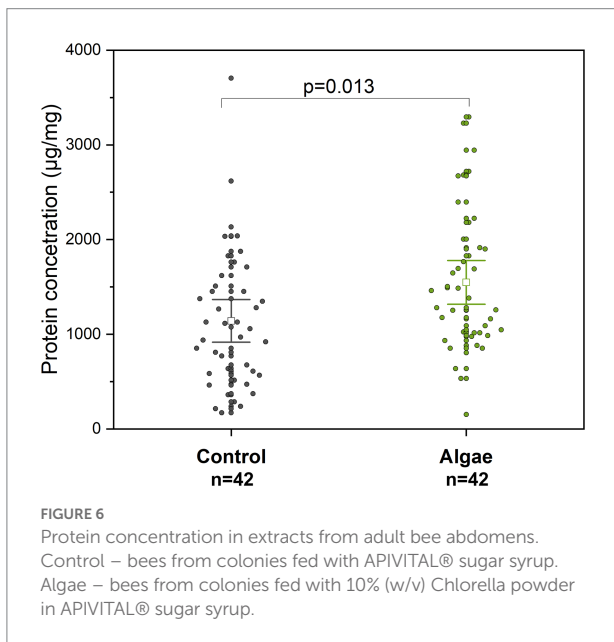
From our results, it is evident that there is no significant impact of Chlorella supplementation in the doses used on the digestive system of bees, with no differences found in midgut mass, protease, amylase activity, and lipase activity. We expected higher protease activity in midguts after Chlorella applications due to the higher amount of proteins in alga feed. Previous research on protein supplements detected higher protease activity in bees fed with high concentration of crude protein in a supplement compared to rape pollen ([Li et al., 2012](#)). This fact is explained by [Moritz and Crailsheim \(1987\)](#) who found correlation between the amounts of protein in the

endo-peritrophic space and protease activity (i.e., trypsin-like and chymotrypsin-like). Pollen grains are enveloped by a polysaccharide protective wall which is probably connected to complicated digestibility and subsequent lower amount of soluble proteins ([Moritz and Crailsheim, 1987](#)). Therefore our findings showed at least similar capacity of proteolytic degradation in midguts of Chlorella supplemented bees compared to non-Chlorella control. Interestingly, lipase activity was slightly but not significantly reduced in the midgut of the Chlorella fed bees, in agreement with previous research ([Jehlík et al., 2019](#)). [Corby-Harris et al. \(2019\)](#) detected higher lipase activity in bees deprived of pollen. Pollen-fed bees had lower activity of lipases suggesting that Chlorella is a suitable source of nutrition. Additionally, it has been described that organic extracts from microalgae have antimicrobial activity. Hence, microalgae-based feed could have a prophylactic properties for honey bee colonies ([Catarina Guedes et al., 2011](#); [Salem et al., 2014](#); [Dostálková et al., 2021](#)).

Metabolic processes in aerobic organisms, including insects, are associated with the production of low physiological levels of ROS which can be efficiently regulated by the enzyme and non-enzyme



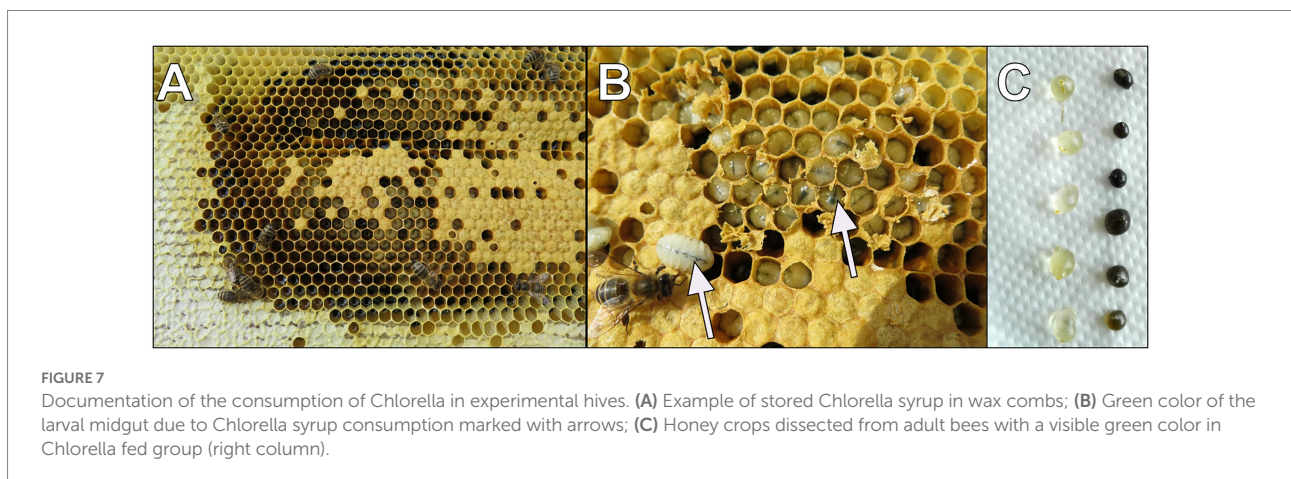
components of antioxidant system (Felton and Summers, 1995; Corona and Robinson, 2006; Sies et al., 2017). Exposure of honey bees to xenobiotic compounds, such as pesticides or herbicides, often results in increased ROS production, with subsequent mobilization of antioxidant enzymes (Chakrabarti et al., 2015; Balieira et al., 2018; Paleolog et al., 2021). When focusing on parameters of oxidative stress in *Chlorella* fed bees, we randomly sampled workers from inside the colony. Our results did not show any significant differences in CAT and SOD enzyme activities in abdomens of control and experimental groups. Based on our results, we could hypothesize that CAT and SOD enzyme activity is sufficient for degradation of ROS in both experimental groups. Alternatively, CAT and SOD does not play significant role in ROS degradation compared to other mechanisms involved in anti-oxidative stress response (Sies et al., 2017).



No differences were also observed in *Chlorella*-fed bees expression patterns of *SOD* and *CAT* genes. Previously, changes in *CAT* and *SOD1* and *SOD2* gene expression levels after feeding bees with a high protein content diet were described by Li et al. (2014). The authors observed that elevated gene expression of antioxidant genes can positively affect the longevity of adult honey bees. A higher expression rate of *CAT* was also observed for colonies surrounded by non-agricultural forage (Ricigliano et al., 2019). No significant difference was recorded for *CAT*, *SOD1*, and *SOD2* gene expression in larvae, despite showing green material in their digestive tracts. Larvae are fed with *Chlorella* indirectly, nurse bees mix royal jelly, a product of hypopharyngeal glands of workers, with *Chlorella* similarly as pollen (Crailsheim et al., 2013). Consequently the concentration of *Chlorella* in larval food is probably decreased. Larval food additionally contains other components such as honey, which is known for its antioxidant properties (Erejuwa et al., 2012).

Both groups of colonies were kept in one apiary with the same opportunities to forage. An insignificantly higher protein (1.4-fold) content was observed in abdomens of adult workers fed by alga diet. This finding is likely indicative of the much higher protein content in alga (about 50% in dry powder) than that in common pollen (7–35%) (data from the Centre Algatech, Institute of Microbiology CAS, Třeboň, Czech Republic). Supplementation of colonies with easy digestible protein-rich diet induces higher protein production in honey bees (Paiva et al., 2019). Higher total protein concentrations in hemolymph in September before wintering is positively correlated with the population size of colonies in the following spring (Smart et al., 2019). Thus, feeding colonies with soluble protein-based food, could increase the protein concentration in honey bee hemolymph and improve the fitness of colonies (De Jong et al., 2009; Morais et al., 2013; Watkins de Jong et al., 2019).

Our results are in agreement with the expected positive impact of *Chlorella* food importantly on the Vg protein level. Previous studies found that Vg production is stimulated by a



protein-rich diet and increased levels of *Vg* are connected with extended longevity (Bitondi and Simoes, 1996; Havukainen et al., 2013; Aurori et al., 2014) and successful overwintering at the colony level (Smart et al., 2019; Ricigliano and Simone-Finstrom, 2020). We detected a 3-fold higher concentration of *Vg* in hemolymph of *Chlorella* fed bees which we attribute to the large number of nutrients that algae contain.

In previous laboratory studies, consumption of algal-based food by bees positively stimulated *Vg* gene expression in adult workers compared to plain sucrose food (Ricigliano and Simone-Finstrom, 2020; Jang et al., 2022). Elevated *Vg* expression corresponded to the pollen rich diet, therefore we conclude that in our study where bees had access to stored or naturally occurring pollen, no differences in *Vg* expression indicate similar effect as pollen or algal-based food consumption as *in vitro*. Jehlík et al. (2019) showed significantly higher *Vg* gene expression after *in vivo* application of *Chlorella* in winter, we explain these differences by various tissues used for the determination of gene expression. In the study of Jehlík et al. (2019) only honey bee heads were used for RNA extraction compared to whole bee body RNA extraction in the present study as we assumed *Vg* expression in the abdominal fat body (Corona et al., 2007). Production of larval *Vg* and even *Vg* gene expression was not influenced in the *Chlorella*-fed group; we have not any satisfactory explanation for this, because a real role of *Vg* in larvae is not fully understood.

Application of *Chlorella*-based pollen supplements represents an attractive measure in the current beekeeping practice. It has the potential to replace the traditional pollen supplements prepared from soya, yeast, wheat, or lentils. Although, the importance of ROS production and its regulation by antioxidant system in maintaining balanced bee homeostasis has been recognized; the actual knowledge on the effects of bee food supplements to ROS production is very limited. *Chlorella*-based supplements have been previously studied from the point of improving colony fitness or effects to nutritional markers such as levels of vitellogenin. Collectively, our results corroborate the expected positive effects of *Chlorella*-supplemented syrup to bee nutritional status assessed as the protein content in adult bees. Additionally, we brought the first evidence that feeding *Chlorella* to honey bee colonies does not elicit increased ROS levels. Further studies should be directed to evaluations of optimal formulations, dosages and timing of *Chlorella*-based supplements. More detailed studies on molecular mechanisms of the digestion and utilization of algal cellular components by different developmental stages and casts of bees are also necessary. The results should provide a solid foundation and guidelines for further implementation of *Chlorella* as an effective and safe pollen substitute in apiculture practice.

## Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

## Author contributions

JD, MS-F, DK, MP, SD: conceptualization. JD, DK, MS-F: methodology. SD, JD, and DK: performed the analysis and data collection. JD and SD: data curation and writing—original draft. SD: visualization. MP and DK: supervision. MP: project administration. DK, MSF, and MP: writing—review and editing. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Supplementary material

The Supplementary material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fevo.2022.1028037/full#supplementary-material>

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