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RESEARCH

Spent coffee grounds extract: antimicrobial activity against *Paenibacillus larvae* and its effect on the expression of antimicrobial peptides in *Apis mellifera*

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Abstract

In recent years, natural alternatives have been sought for the control of beekeeping pathologies; in the case of American Foulbrood (AFB) disease, the use of synthetic antibiotics was prohibited due to honey contamination and the generation of resistant bacteria. The significant increase in population growth worldwide has led to great concern about the production of large amounts of waste, including those from agribusiness. Among the most important beverages consumed is coffee, generating thousands of tons of waste called spent coffee grounds (SCG). The SCG is a source of many bioactive compounds with known antimicrobial activity. The aims of the present work were: (1) to obtain and chemically analyse by HPLC of SCG extracts (SCGE), (2) to analyse the antimicrobial activity of SCGE against vegetative form of *Paenibacillus larvae* (the causal agent of AFB), (3) to evaluate the toxicity in bees of SCGE and (4) to analyse the effect of the extracts on the expression of various genes of the immune system of bees. SCGs have a high content of phenolic compounds, and the caffeine concentration was of 0.3%. The MIC value obtained was 166.667 µg/mL; the extract was not toxic to bees, and interestingly, overexpression of abaecin and hymenoptaecin peptides was observed. Thus, SCGE represents a promising alternative for application in the control of American Foulbrood and as a possible dietary supplement to strengthen the immune system of honeybees. Therefore, the concept of circular bio-economy could be applied from the coffee industry to the beekeeping industry.

Keywords American foulbrood · *Apis mellifera* · Immunity genes · *Paenibacillus larvae* · Spent coffee ground extract

Introduction

During the last years, there has been an increase in the use of extracts derived from agricultural by-products in the pharmaceutical industries due to the presence of antibiotic resistant bacteria (Valgas et al. 2007). In the case of beekeeping, in addition to antimicrobial resistance, the constant use of synthetic antibiotics for the control of American foulbrood, a disease caused by the Gram-positive bacterium *Paenibacillus larvae*, leads to a potential persistence of chemical residues in honey, limiting its commercialization for human consumption, in addition to negative effects on development and survival of bees (Genersch 2010). It has been observed in in vitro tests that different natural extracts had antimicrobial activity against vegetative form of *P. larvae* (Eguaras et al. 2005; Fuselli et al. 2006, 2010; Giménez-Martínez et al. 2019, 2020, 2021). On the other hand, there is the possibility of remaining chemical residues in honey, limiting its commercialization for human consumption.

In recent years, great importance has been given to the potential use of waste generated by the industry in order to give it considerable economic value (Chiocchio et al. 2021). The organization Rethinking food waste through economics and Data (ReFED 2016), identified around 27 possible solutions grouped into: (1) prevention, (2) recovery, and (3) recycling (ReFED 2016). Thanks to these analyses, the idea of a circular economy began to form,

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using the reuse of agro-industrial waste, thus minimizing pollution, carbon emissions, etc. (Barreiro-Gen and Lozano 2020; Morseletto 2020).

One of the most popular beverages worldwide is coffee (Jung et al. 2012), its consumption has steadily increased over the years, generating a large amount of solid waste such as spent coffee grounds (SCG) (Banu et al. 2020). World coffee exports amounted to 11.4 million bags in February 2022, and particularly in Argentina, 640.000 bags of cake of 60 kg each were imported for consumption during 2021 (http://www.ico.org). Coffee brewing at homes and gastronomic establishments consists of extracting select compounds from the grain, therefore this industry generates massive organic waste, like SCG; the economic and environmental costs to dispose of SCG are undesirable, which is why alternatives are being sought for its use (Mc Nutt 2019).

SCGs extracts have a high content of phenolic compounds, among the main ones are hydroxycinnamic acids and flavonoids (Murthy and Naidu 2012); these compounds have important antiviral, antimicrobial, and anti-inflammatory activity, among others (Esquivel and Jimenez 2012). Several studies demonstrate the antioxidant and antimicrobial activities of coffee by-products (Duangjai et al. 2016; Moreira et al. 2018; Mirón-Mérida et al. 2019); for example, Monente et al. (2015) observed that both coffee and the extracts obtained prevented the growth of pathogenic microorganisms in food.

In recent years, environmental awareness has increased worldwide, which has led to legislation on agro-industrial waste, providing high added value within the framework of a circular bioeconomy (Kourmentza et al. 2018), the aims of the present work were: (1) to obtaining and chemically analyse by HPLC of SCG extracts (SCGE), (2) to analyse the antimicrobial activity of SCGE against *P. larvae*, (3) to evaluate the toxicity in bees and (4) to gain a more comprehensive understanding of the impact of SCGE, we analyse the effect of the extracts on the expression of genes related to the immune system of bees.

Materials and methods

Biological material

Reference strains of *P. larvae* (ATCC 9545) and strains of *P. larvae* UB-CIDEFI PL92 and PL62 were used in all the experimental tests, the three strains were ERIC 1 (Fuentes et al. 2023), and were acquired from the OIE Reference Laboratories for AFB. Strains were maintained on MYPGP agar (Mueller–Hinton broth 10 g/l^{-1}, yeast extract 15 g/l^{-1}, K_2HPO_4 3 g/l^{-1}, glucose 2 g/l^{-1}, sodium pyruvate 1 g/l^{-1}, and agar 20 g/l^{-1}) and stored at 4 °C. For long-term maintenance, the strains were stored at − 20 °C in J broth (15 g/l^{-1}yeast extract, 5 g/l^{-1}tryptone, 3 g/l^{-1}K_2HPO_4 and 2 g/l^{-1}glucose) with the addition of 20% v/v glycerol.

Adult honeybees used are the so-called “criollas”, which are the result of the cross between *A. mellifera* carnica and *A. mellifera* ligústica. The apiary was located on the outskirts of Mar del Plata city (37°50×04”S; 57°37×42”W) and in Santiago city (33°23’26.7”S 70°30’02.8”W).

The SCG extraction method and HPLC analysis

SCG of variety arabica obtained from restaurants was dried for 48 h at room temperature and stored at 4 °C until further analysis. The SCG extract (SCGE) has been obtained using the Soxhlet method, briefly, 25 g of SCG was dissolved in 300 mL of ethanol, and the organic fraction was obtained by four Soxhlet cycles (Bouhlal et al. 2020). The extract was resuspended on ethanol/water (50:50) to a final concentration of 2 mg/mL.

The analysis of the phenolics compounds and caffeine content was performed by HPLC. The phenolics compounds analyzed were: coumaric acid, catechin, epicatechin, quercetin, gallic acid, and ferulic acid, using HPLC (HPLC Infinity 1260, Agilent Technologies, Inc) (UV detector, Column C18 reverse phase 4.6×75 mm; the mobile phase was acetic acid:methanol [75:25]).

Analysis of antimicrobial activity: broth microdilution technique

The antimicrobial activity of the SCGE was determined by the broth microdilution method (Pellegrini et al. 2017). Twofold serial dilutions ranging from 666.667 to 5.2083 µg/ml of the SCGE were performed. Bacterial strains were routinely grown and maintained on MYPGP-agar plates (described in Sect. 2.1) and incubated at 37 °C and 10% (v/v) CO_2 for 48 h. The bacterial inoculum was prepared in sterile peptone H_2O (peptone 0.001 g/ml^{-1} and NaCl 0.0085 g/ml^{-1}) to a final optical density at 600 nm (OD600nm) of 0.1 using a spectrophotometer T60UV/VIS Spectrophotometer Labindia (New Bombay, India). Brain-heart infusion (0.037 g/ml^{-1}) was used as a growth media for the bacterial strains when performing the broth microdilution assay. Bacterial growth was detected after incubating the plates for 1 h with resazurin sodium salt at 0.0001 g/mL^{-1} (10 µL), a blue redox indicator that turns pink in the presence of aerobic growing bacteria.

The percentage of inhibition corresponding to the MIC visually determined was calculated using the followed equation :

$$Inhibition(\%) = \left(\frac{n^{\circ}colonies\ in\ control - n^{\circ}colonies\ in\ sample}{n^{\circ}colonies\ in\ control} \right) * 100$$

based on the number of viable colonies present in wells corresponding to the visual MIC (sample) and in control wells with bacteria only (control), for this purpose 100 µL of culture were seeded directly or from serial dilutions on

MPYG agar plates and incubated for 48 h. Subsequently, the number of colonies was quantified and the dilution factor was considered.

The concentration at which vegetative form of *P. larvae* growth was inhibited by 90% at 48 h was considered the Minimum Inhibitory Concentration (MIC) (Dingman and Stahly, 1983). Antimicrobial activity assays were performed in triplicate (technical replicates) and repeated for 3 consecutive days (biological replicates). The solvent ethanol:water (50:50) was used as a negative control to observe if it had any influence as antimicrobial agent.

Toxicity test on *A. mellifera*

The toxicity test of SCGE against adult *A. mellifera* was performed using the complete exposure test (Ruffinengo et al. 2005). The doses used per capsule were 2 mg/mL; 2 mL of the extract solution was placed on the base of a petri dish. The extract and solvent were evaporated at room temperature, and then worker bees were placed on the petri dish. The petri dishes were incubated at 30 °C and 70% relative humidity. The mortality of the bees was evaluated at 24 and 48 h after the start of the trial (OECD 1998). For the assay, three repetitions of ten bees each were carried out, both for SCGE and for the control group (ethanol: water (50:50).

For the evaluation of SCGE toxicity in *A. mellifera* larvae, the administered diet contained 50% of royal jelly, obtained commercially, and 50% of sugar solution composed of yeast extract, D- glucose (Sigma-Aldrich, St. Louis, MO, USA) and D-fructose (Fluka, St. Gallen, Switzerland) (Aupinel et al. 2005). The larvae used were those that were in stage L1, on day four of incubation (stage L4), was administered in the diet the SCGE at 166,667 µg/mL (MIC value of SCGE against *P. larvae* vegetative cells), larvae mortality was recorded at 24, 48 and 72 h. All larvae received a total of 160 µL of diet for 6 days. A control group was considered whose diet contained only the solvent used in the extraction (ethanol:water (50:50). Thirty-two larvae were cultured per group. Incubation conditions were 34 ± 1 °C and 95% RH (OECD 2013). Likewise, the mobility of the larvae was compared day by day among the different groups.

Gene expression analyses

The test was carried out with bees that emerged in the laboratory; they were caged and kept consuming syrup for 24 h. On day 2, one group of bees was exposed to a final concentration of 0.02 mg/mL of SCGE dissolved in syrup for 24 h (from day 2 to day 3). On day 3, bees were given syrup and on day 4, bees were removed for expression analysis. Together with the exposed group, a control group was carried out with bees only exposed to syrup. Each experimental condition was tested in forty bees (ten bees per cage), and five bees were used in each group for RNA extraction. The other bees were used to calculate food consumption and bee survival.

RNA extraction

Total RNA was extracted from abdomen using RNAeasy mini kit (Qiagen, Hilden, Germany) according to the supplier's instructions. RNA quantification was determined using the NANODROP 2000 kit (Thermo Scientifics, USA).

DNase treatment

DNA was removed from samples using DNase I, Amp Grade (Ambion, life technologies, USA). 1 µg/µL of RNA, 16 µL of RNase-free water, 2 µL 10X DNase I reaction buffer (200 mM Tris-HCl (pH 8.4), 20 mM $MgCl_2$, 500 mM), and 1 µL of DNase I AMp Grade was added (1 Unit/µL). The enzymatic reaction was carried out at 25 °C for 15 min, and then the temperature dropped to 4 °C where 2 µL of 25 mM EDTA (pH 8.0) was added and finally, it was incubated at 65 °C for 10 min. We corroborated the removal of contaminating DNA after treatment with DNase I by verifying the non-amplification of β-actin prior to cDNA synthesis. The samples were stored at − 20 °C until later use.

Reverse transcription

For a final volume of 20 µL, 5 µL of total RNA treated with DNase I was added, and then a first mix was added consisting of 5 µL of nuclease-free water and 2 µL of oligo (dT) 12–18 500 µg/mL. It was incubated at 70 °C for 10 min and then the second mix consisting of 4 µL of 5X First-Strand buffer (250 mM Tris-HCL (pH 8.3), 375 mM KCl, 15 mM $MgCl_2$), 2 µL of 10 mM dNTPs, 1 µL of RNasin® Ribonuclease Inhibitor, Promega (40 units/µL), and 1 µL (200 units/µL) of MMLV RT (Invitrogen, USA), finally incubated at 42 °C for 50 min and then at 70 °C for 15 min. The cDNA was stored at 4 °C until further use.

Real-time PCR

The reaction mixture with a final volume of 10 µL consisted of 1 µL of cDNA, mixed with 5.8 µL of H_2O, PCR-grade, 1.2 µL of 25 mM $MgCl_2$, 0.5 µL of each primer (Table 1) at a concentration of 10 µM, and 1 µL of 10X Master SYBR Green I in Light Cycler thermo cycler kit (Roche, Indianapolis, IN). Amplification was performed with a cycle of pre-incubation at 94° C for 10 min, followed by 40 cycles at 95 °C for 10 s, 55 °C for 10 s, 72 °C for 15 s, and a final cycle at 65 °C for 15 s. The amplicons were characterized according to their melting temperature, determined in the LightCycler

Table 1 Primer sequences used for real-time PCR analysis

Primers	Sequence (5'◊3')	Amplification Target	Reference	Real-Time Efficiency (this study)
β-actin-F β-actin-R	TGCCAACACTGTCCTTTCTG AGAATTGACCCACCAATCCA	β-Actin (reference gene)	Yang and Cox-Foster (2005)	2.00
Abaecin-F Abaecin-R	CAGCATTCGCATACGTACCA GACCAGGAAACGTTGGAAAC	Antibacterial peptide abaecin	Evans et al. (2006)	2.00
Defensin-F Defensin-R	TGCGCTGCTAACTGTCTCAG AATGGCACTTAACCGAAACG	Antibacterial peptide defensin	Yang and Cox-Foster (2005)	1.92
Hymenopt-F Hymenopt-R	CTCTTCTGTGCCGTTGCATA GACCCACCAATCCATACGGA	Antibacterial peptide hymenoptaecin	Evans et al. (2006)	1.94
Dorsal 1-F Dorsal 1-R	AGAGATGGAACGCAGGAAAC TGACAGGATATAGGACGAGGTAA	NFκB transcriptor factor	Tesovnik, (2017)	1.99
PGRPLC710-F PGRPLC710-R	TCCGTCAGCCGTAGTTTTTC CGTTTGTGCAAATCGAACAT	Peptidoglycan recognition protein LC	Evans et al. (2006)	1.98
Relish-F Relish-R	GCAGTGTTGAAGGAGCTGAA CCAATTCTGAAAAGCGTCCA	NFκB transcriptor factor	Evans et al. (2006)	2.00
Toll-F Toll-R	TAGAGTGGGGCATTGTCAAG ATCGCAATTTGTCCCAAAAC	Transmembrane protein receptor	Evans et al. (2006)	2.00

equipment, and the sizes were verified by agarose gel electrophoresis.

Quantitative real-time PCR

The quantification of the relative expression of the mRNA was performed using comparative Ct method $2^{(-\Delta\Delta Ct)}$ (Schmittgen and Livak 2008). The Ct (threshold cycle), obtained for the genes of interest was normalized using the Ct obtained for β-actin (constitutive gene), after verifying the amplification efficiency of each gene with respect to β-actin by performing a standard curve. The amplification efficiency was determined with the following formula: $E = 10^{(-1/m)}$ where E corresponds to the amplification efficiency and m to the slope of each standard curve.

Statistical procedures

Regarding toxicity in *A. mellifera*, a dose-response analysis was performed by applying the Mantel-Cox Test to compare the concentrations used in each test. A Chi^2 test was performed to compare mortality between the experimental group and the control group and to assess whether the SCGE is a no observed adverse effect level (NOAEL) (Medrzycki et al. 2013).

Larvae and bees survival rate was analyzed by a Kaplan-Meier survival method for control and SCGE groups, and significant differentiation was determined by the log-rank Mantel-Cox test. To determine differences between control and SCGE groups on syrup consumption, we performed the Shapiro-Wilk normality test data distribution and then, a two-way ANOVA was performed followed by Bonferroni's *post-hoc*, $P < 0.05$.

We performed the Shapiro-Wilk normality test data distribution to analyze the effect of SCGE on gene expression. After the determination of non-normality distribution, a non-parametric Mann-Whitney U -test, was used to determine significant differences in the pairwise comparison of experimental groups. For all the statistical analyses, the software GraphPadPrism version 7.00 for Windows, GraphPad Software, La Jolla California USA, http://www.graphpad.com was used for analysis.

Results

Chemical characterization and antimicrobial activity of SCG extract

SCGs are valuable residues due to their high content of phenolic compounds and antioxidants, such as chlorogenic acid, caffeine, and various flavonoids (Mussato et al. 2011).

The extract contained 0.3% (2012.567 ppm), Fig. 1 shows the HPLC analysis for caffeine concentration, phenolic compounds found were only epicatechin and ferulic acid. The major component was epicatechin, this compound represents 40% of the total analysis, and its concentration was 135.06 ppm (Figs. 2 and 3). Future studies will focus on the analysis of more compounds to perform a more complete chemical profile.

When analyzing the antimicrobial activity of SCGE, a promising MIC value was observed, being 166.667 µg/mL for the three *P. larvae* strains tested.

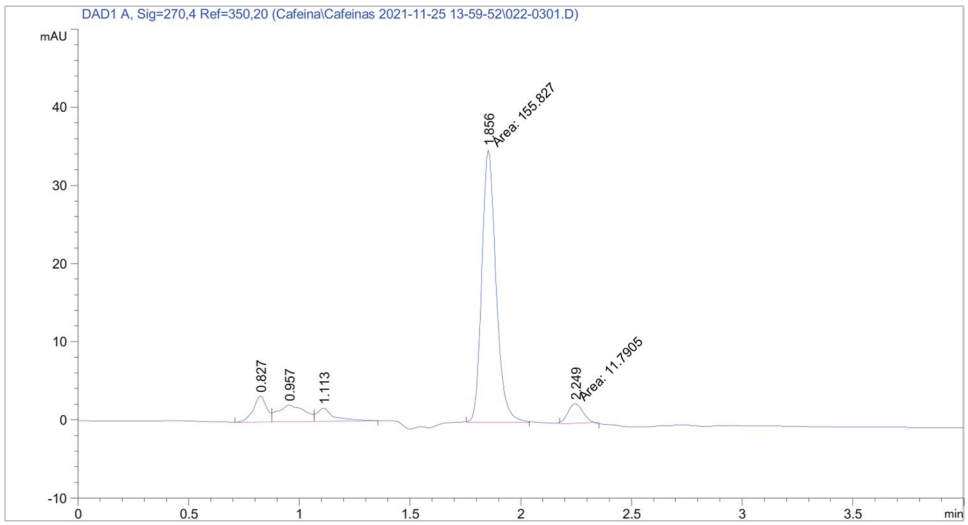

Fig. 1 HPLC analysis for caffeine concentration. The y-axis of the chromatogram is a measure of the intensity of absorbance (in units of mAU), and the x-axis measure the retention time (minutes)

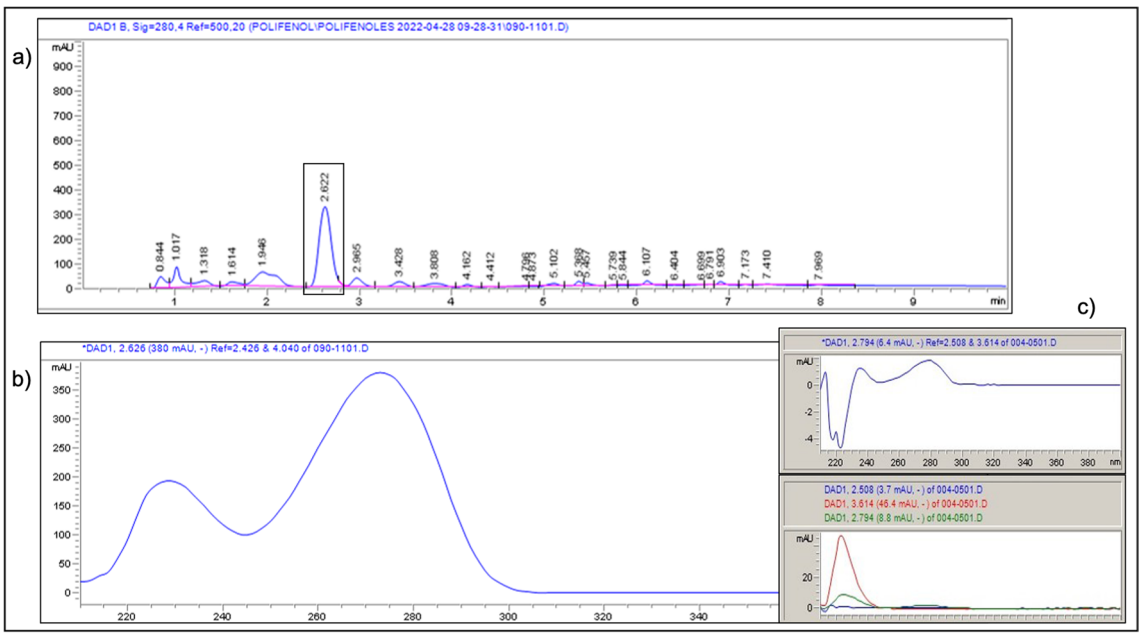

Fig. 2 HPLC analysis of SCGE, **a** The rectangle marks the peak of epicatechin. **b** The shape of the peak is observed, where it is compared with the standard for epicatechin (**c**). The y-axis of the chromatogram is a measure of the intensity of absorbance (in units of mAU), and the x-axis measure the retention time (minutes)

Toxicity of SCGE in *A. mellifera*

The toxicity test showed that SCGE was not toxic to bees. Mortality did not exceed 6% when compared to the control group and it was not significant (Fig. 4). The Chi2 test did not give significant differences (X2: 1.999, df: 1, p-value: 0.1574), therefore the SCGE was considered NOAEL.

Likewise, the acute exposure of *A. mellifera* larvae to a concentration of SCGE corresponding to the MIC against *P. larvae* vegetative cells had no toxic effect, with a mortality rate not exceeding 10%. (p-value: 0.5699). (Fig. 5)

Effect of SCGE on immune related gene expression of honey bees

The data also showed that there is no significant difference in the mortality, after 6 days of exposure. Mortality close to 40% was observed for SCGE and less than 20% for the control group (p-value: 0.0838) (Fig. 6).

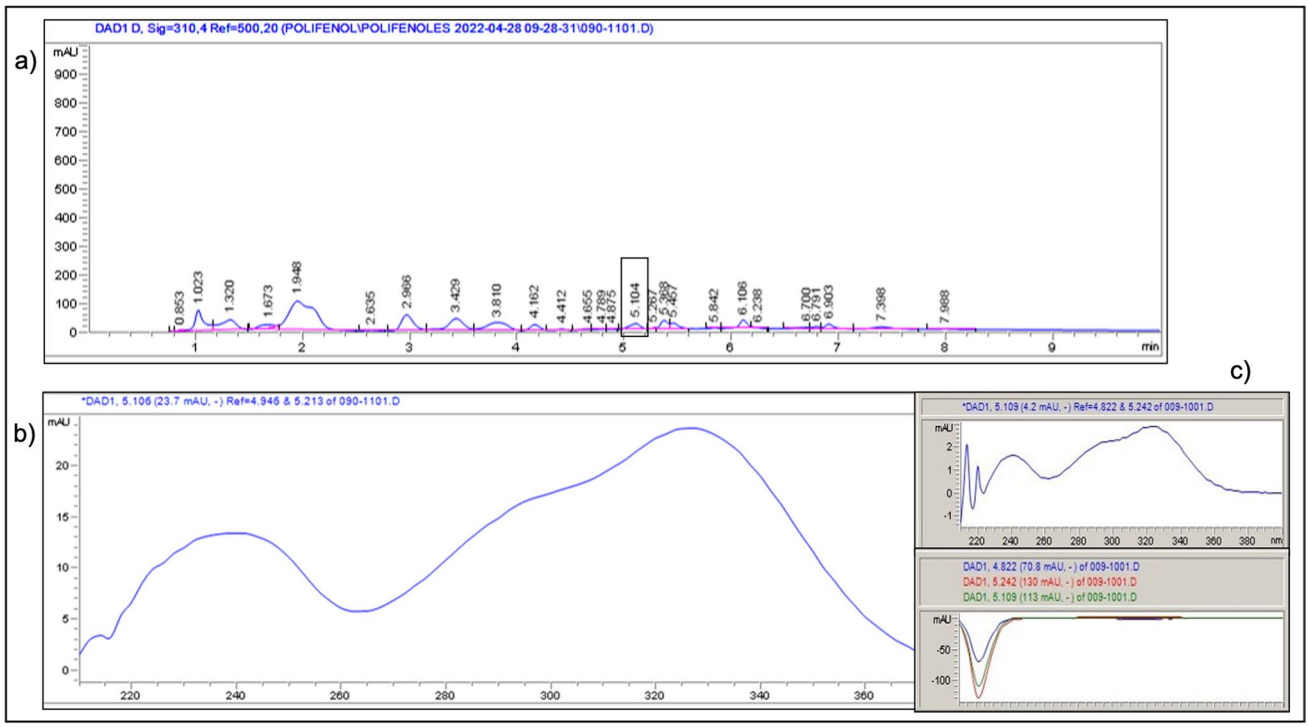

Fig. 3 HPLC analysis of SCGE, **a** the rectangle mark the peak of ferulic acid, **b** the shape of the spectrum obtained is observed, and (**c**) the ferulic acid standard spectrum. The y-axis of the chromatogram is a measure of the intensity of absorbance (in units of mAU), and the x-axis measure the retention time (minutes)

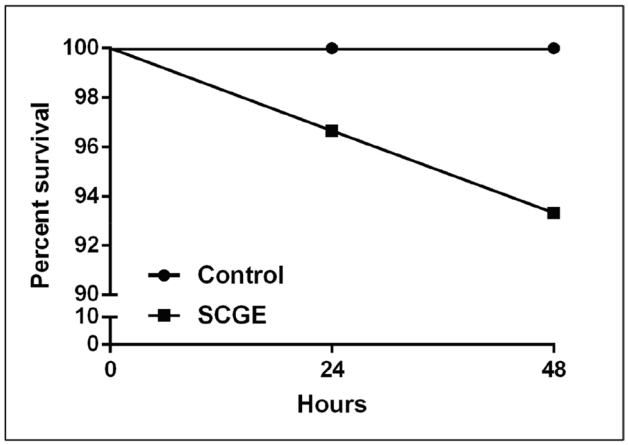

Fig. 4 Toxicity analysis in bees fed with SCGE. To analyse the toxicity, the Mantel-Cox Test was used

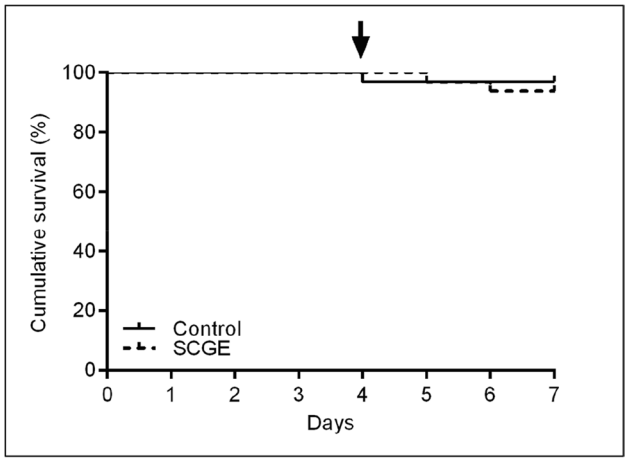

Fig. 5 Kaplan-Meier survival curve of the experimental honey bee larvae (*Apis mellifera*), no treated (control), or in response to SCGE exposure. The arrow indicates the acute exposure day to the treatment

Regarding syrup consumption, there were no significant differences between the groups analysed (Fig. 7).

We chose genes encoding honey bee antimicrobial peptides (abaecin, defensin-2, hymenoptaecin) and genes whose expression is related to the Toll signaling pathway (Dorsal 1, Toll), and the IMD signaling pathway (PGRP-LC, Relish), both of which mediate immunity against pathogens in *A. mellifera.*

In SCGE-fed honey bees, there were only two genes that were overexpressed, abaecin and hymenoptaecin (p-value < 0.05), when compared to the control at 48 h post-exposure (Fig. 8a and c). Defensin-2, Dorsal-1, PGRP-LC, and Relish genes did not show significant changes in their expression.

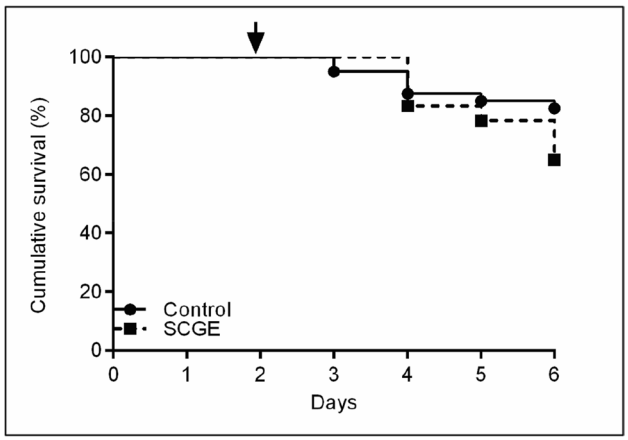

Fig. 6 Kaplan-Meier survival curve of the experimental adults honey bee (*Apis mellifera*), no treated (control), or in response to SCGE exposure in sucrose syrup. The arrow indicates the acute exposure day to the treatment

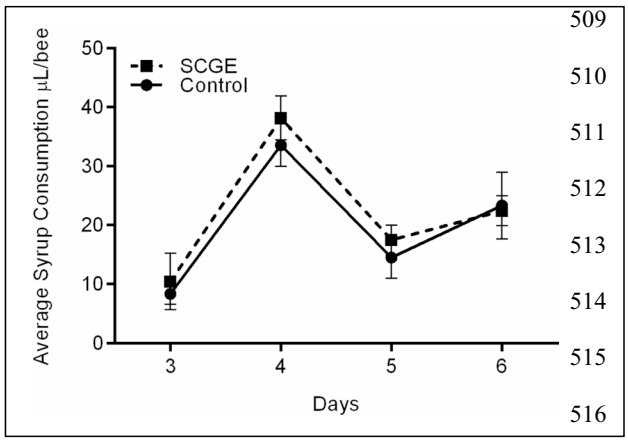

Fig. 7 Average daily consumption of syrup in the control and SCGE groups from day 3 up to 6

Discussion

There are few publications reporting the antimicrobial activity of SCGE; Monente et al. (2015) and Bouhlal et al. (2020) carried out a screening of antimicrobial activity, and they observed that it was effective against gram-negative and gram-positive bacteria. Although there are few publications on the antimicrobial activity of SCG extracts, there is a lot of literature on the bioactivity of phenolic compounds (Zhang et al. 2022). For example, Mewaba et al. (2022), analysed the antimicrobial activity of compounds isolated from coffee, where they observed that the MIC obtained for caffeine was > 512 µg/mL against methicillin resistant *Staphylocccus aureus* and clinical strains.

The mechanism of action of these organic compounds is by altering the structure and function of the cytoplasmic membrane, interrupting the proton motive force, and electron flow, among others (Almeida et al. 2006).

Perumal et al. (2017, 2018) analysed the mechanism of action of epicatechin, the main component present in our extract against *Pseudomonas aeruginosa*. First, they analysed the permeability of the outer and plasmatic membrane, observing that it increased, allowing access to other phenolic compounds; in turn, when analysing the integrity of the cell surface, they observed that the bacteria appeared shorter and the cell surface structure more corrugated.

Regarding the effect of phenolic compounds on the expression of immune system genes in insects and, in particular, in bees, there is little literature. After analysing honey extracts, Mao et al. (2013) found that p-coumaric acid caused overexpression of detoxification genes and those responsible for defense against pathogens (abaecin and defensin-1). On the other hand, Shi et al. (2020) observed that after short-term exposure to quercetin in *Bombyx mori*, defensin mRNA gene levels were significantly higher than in the control group.

Our results agree with those obtained by the authors mentioned above in the sense that phenolic compounds show an effect on the expression levels of immunity genes.

The study by Lu et al. (2020) contrasts with our results, they reported that abaecin had a slight increase in relative expression however, defensin-2 e hymenoptaecin expression was not modified. This discrepancy can be explained by the different extracts used. Lu et al. (2020) used pure caffeine at 0.1 mM (0.019 mg/mL), and we used a spent coffee ground extract at 0.02 mg/mL, which has several compounds in its chemical composition, including caffeine in low proportion, it only represents 0.3% of SCGE. Our results show that the compounds present in our SCGE favor the expression of antimicrobial peptides. This could suggest that SCGE consumption would protect bees against pathogens.

In most cases, synergistic antimicrobial action of bioactive component present in plant extracts has been described (Hajimehdipoor et al. 2014). This enhanced effect with respect to their individual components is mainly associated with the fact that bioactive molecules inhibit bacterial growth in various ways by affecting the structure and integrity of the plasma membrane, permeability or functionality. (Savoia 2012; Radulovic et al. 2013)

It would be interesting to focus future studies on analyzing the effect of the main SCGE compounds separately and together, to further understand their mechanism of action against *P. larvae* and in its effect on gene expression, since it was previously observed that both phenolic compounds p-coumaric acid and caffeine have a positive action on gene expression (Mao et al. 2013, Lu et al. 2020), in honey bees.

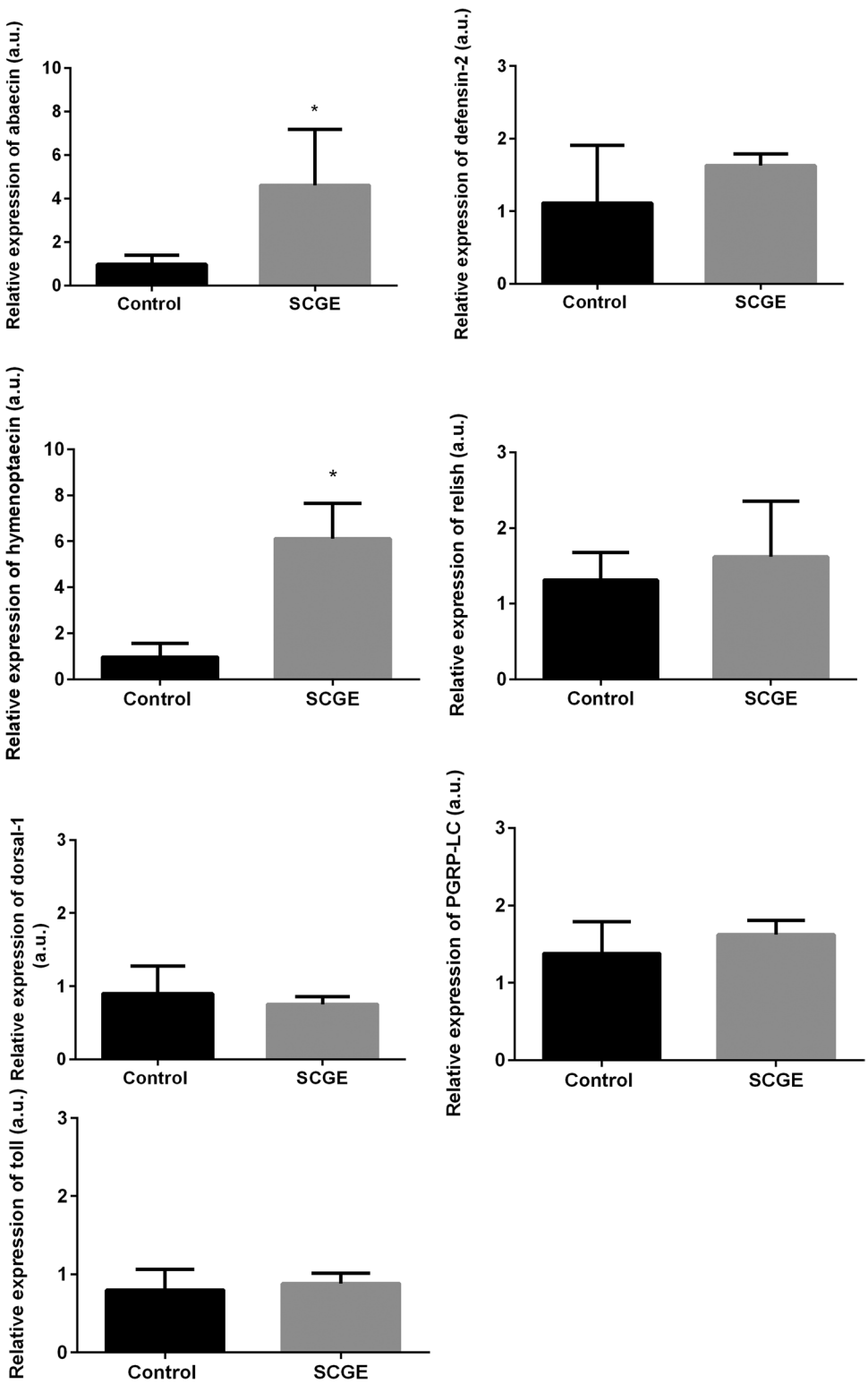

Fig. 8 Relative expression of genes in honey bees fed with SCGE. The asterisk indicates a significant difference (p-value < 0.05). To analyse the gene expression, the Shapiro-Wilk normality test data distribution and Mann-Whitney U-test were performed, and a non-parametric test was used to determine significant differences in pairwise comparison

Another aspect to considerate is the study of potential benefits of SCGE consumption on honey bee health.

For future applications of SCGE as an antimicrobial agent against *P. larvae*, there are some inherent challenges to realize the translation of in vitro assay to the use of SCGE in hives, for example, different varieties of coffee residues should be used for future trials and to determine which of these extracts has the best antimicrobial activity an evaluate the reproducibility of the composition of SCGE. Accurate characterization of bioactive compounds is necessary to be

established quality control procedures, and standardization of the extract.

Conclusion

The discharge of SCG produces 28.6 million tons of CO_2 eq per year, so it is necessary to manage the disposal of SCG to reduce its environmental impact. With the recent stimulation of a circular bio-economy, SCG has begun to be revalued, such as obtaining bio-compounds.

The aim of our study was to evaluate the use of coffee waste extracts as a possible antimicrobial agent against *P. larvae*, the causative agent of AFB, one of the most serious diseases affecting honey bees. Along with this, we wanted to evaluate immunological parameters such as the expression levels of genes that participate in the immune response.

Our results show that SCGE has antimicrobial activity against *P. larvae* vegetative cells, is not toxic against *A. mellifera*, and can induce the overexpression of genes encoding for antimicrobial peptides the most important defence system in honey bees; both aspects are relevant given that finding antimicrobial alternatives to antibiotics and improving the immune system of bees is of worldwide interest.

Our results suggest that the reuse of SCG would mitigate a major problem such as the final elimination of this abundant waste, the global production of coffee reached more than 9.513 billion kg in 2017–2018, the economic and environmental costs to dispose of SCG are undesirable, which is why alternatives are being sought for its use.

Likewise, it will be interesting to evaluate in future research the antimicrobial activity of the SCGE against *P. larvae* spores, which would favor its possible use in the control of American foulbrood. Furthermore, these results suggest the possibility of improving the immune response of honey bees, all of which would result in the strengthening of the colony.

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Author contributions PG: designed, planned, and conducted experiments; analysed data; and wrote the original draft. FZ performed quantitative real-time PCR and laboratory honey bee experiments for gene expression analysis. SM conducted experiments and review the manuscript. SF conceived the project, secured funding, and review the manuscript. JM conceived the project, designed the study and experiments, analysed data, secured funding, reviewed, and edited the manuscript. All authors have read and agreed to the publishing version of the manuscript.

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Data availability All datasets used and/or analyzed during this study are included in this article and are available from the corresponding author on reasonable request.

Declarations

Ethical approval No approval of research ethics committees was required to accomplish the goals of this study because experimental work was conducted with an unregulated invertebrate species.

Competing interests The authors declare no competing interests.

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