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DIET SUPPLEMENTATION HELPS HONEY BEE COLONIES IN COMBAT INFECTIONS BY ENHANCING THEIR HYGIENIC BEHAVIOUR

Zoran STANIMIROVIĆ[1], Uroš GLAVINIĆ[1]*, Marko RISTANIĆ[1], Stefan JELISIĆ[1], Branislav VEJNOVIĆ[2], Mia NIKETIĆ[1], Jevrosima STEVANOVIĆ[1]

[1]University of Belgrade - Faculty of Veterinary Medicine, Department of Biology, Bulevar Oslobodjenja 18, 11000 Belgrade, Serbia; [2]University of Belgrade - Faculty of Veterinary Medicine, Department of Economics and Statistics, Bulevar Oslobodjenja 18, 11000 Belgrade, Serbia

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The hygienic behavior in honey bees is a complex polygenic trait that serves as a natural defense mechanism against bacterial and fungal brood diseases and *Varroa destructor* mites infesting brood cells. The aim of this study was to evaluate the effect of a dietary amino acids and vitamins supplement "BEEWELL AminoPlus" on hygienic behavior of *Apis mellifera* colonies combating microsporidial and viral infections. The experiment was performed during a one-year period on 40 colonies alloted to five groups: one supplemented and infected with *Nosema ceranae* and four viruses (Deformed wing virus - DWV, Acute bee paralysis virus - ABPV, Chronic bee paralysis virus - CBPV and Sacbrood virus – SBV), three not supplemented, but infected with *N. ceranae* and/or viruses, and one negative control group. Beside the listed pathogens, honey bee trypanosomatids were also monitored in all groups.

The supplement "BEEWELL AminoPlus" induced a significant and consistent increase of the hygienic behavior in spite of the negative effects of *N. ceranae* and viral infections. *N. ceranae* and viruses significantly and consistently decreased hygienic behavior, but also threatened the survival of bee colonies. The tested supplement showed anti-*Nosema* effect, since the *N. ceranae* infection level significantly and consistently declined only in the supplemented group. Among infected groups, only the supplemented one remained *Lotmaria passim*-free throughout the study. In conclusion, diet supplementation enhances hygienic behavior of honey bee colonies and helps them fight the most common infections of honey bees.

Keywords: amino acid, vitamin supplement, hygienic behaviour, *Nosema ceranae*, honey bee viruses.

*Corresponding author: e-mail: uglavinic@vet.bg.ac.rs

INTRODUCTION

Not only is the honey bee (*Apis mellifera*) a producer of honey, royal jelly and other bee products but is also the most important insect pollinator of both wild flora and crop species. For this reason, the decline in honey bee abundance which began at the end of the 20th century severely influenced the losses in agriculture [1]. Thus, it is not surprising that increasing efforts are being made to find out all putative factors contributing to the decrease in bee populations [2,3]. Environmental pollutants such as agrochemicals [4-6] and heavy metals [7,8], together with pathogens [9,10], inadequate floral resources [11,12] and incorrect beekeeping practices [2] have been accused of having contributed to the decline in the number of bees. Among pathogens, there are endoparasites capable of compromising bee health and contribute to colony mortality, like microsporidia [13-17] and trypanosomatids [18-20], but also viruses [21-25]. However, they do not necessarily inflict damage per se, but may synergistically influence bees and even lead to colony death [26-30].

Hygienic behavior of honey bees is one of the many strategies they apply to effectively fight pathogens and presents their collective reaction to the presence of diseased brood [reviewed in 31-33]. It results in the capability of the workers to detect the diseased brood and remove the larvae or, later, to open the wax capping of the cells and remove the pupae [reviewed in 34]. By hygienic behavior, considered a social immune response of honey bees, honey bees fight against American foulbrood, chalkbrood, as well as against *V. destructor* [31-33,35-39]. Being highly expressed in *A. cerana* than in *A. mellifera*, hygienic behavior may be one of the reasons underlying better health of the former [40].

Although assessed as low to moderately heritable, with a heritability (h^2) ranging from 0.17 to 0.65 [41-45], hygienic behavior is one of the most common traits selected for breeding programs [45-47]. However, a great number of candidate genes have been associated with hygienic behavior [48-55] reaching as much as 73 genes [54]. Being such highly polygenic, this behavior would be expected to be susceptible to external influences, but the investigations are scarce and the findings non-consistent. Bigio et al. [56] reported no great influence of environmental conditions on hygienic behavior, but a reverse relationship was reported between hygienic behavior expression and altitude [57], seasonal and environmental variations [58]. Hygienic behavior was not correlated with the colony strength [59] and was not affected by *Nosema ceranae* infection [60]. However, the agrochemical imidacloprid was reported to significantly impair hygienic activities of worker bees [61].

Beekeeping practices (colony manipulations, inadequate feeding, chemical and non-chemical 'alternative' treatments, migrations etc.) often negatively affect bee health [reviewed in 2 and 62; 63,64] but little is known about the influence of such activities on honey bee hygienic behavior. Sucrose syrup availability or scarcity and brood manipulation did not significantly change the behavior [56]. Neither of alternative treatments against *Varroa* and/or *Nosema* parasites, e.g. sugar dusting [65], thymol [66],

chitosan and peptidoglycan [60], threatened the bees' hygienic potential, and thymol even increased the uncapping and removal of dead brood [66]. However, migratory beekeeping practice seems to have a negative influence on hygienic behavior [58].

Given that the ecosystem is globally rather devastated, which inevitably leads to obvious lack of high-quality diverse bee forage [11], to meet the needs of the honeybees it is often resorted to the appliance of dietary supplements [2]. Having in mind the increasing use of sugar syrup [67,68] which provides only energy, it is important to also provide mineral, vitamin and protein feed components. Thus, various dietary supplements have been tested for the ability to improve bee health, colony strength, food reserves and productivity [reviewed in 69] including pollen-substitute diets (protein supplements) and protein/vitamin supplements [70-79].

Due to the scarcity of data on the impact of supplementary diet on hygienic behavior of honey bees, the aim of this study was to evaluate the effect of a dietary amino acid and vitamin complex on hygienic behavior of full-sized free-flying colonies and their combat with *N. ceranae* and honey bee-associated viruses. During the study, honey bee trypanosomatids were also monitored knowing their common coexistence with *Nosema* sp. microsporidia [80-82].

MATERIAL AND METHODS

Honey bee colonies

The research began in autumn 2019, on 80 honey bee (*Apis mellifera*) colonies originated from queens selected for hygienic behavior and located on the Pester Plateau (43°16'14" N, 19°59'35" E), Sjenica municipality, Serbia. Colonies were regularly checked for both bee and brood pathology by a veterinary specialist and were without signs of any disease including varroosis and infections caused by honey bee-associated viruses. The absence of *Varroa* infestation was proven by all three methods (debris, brood and bee examinations) recommended in COLOSS BEEBOOK [83]. Prior to wintering, the colonies were supplied with optimum content of natural food resources (ca. 19 kg of meadow honey and two frames filled with bee bread on both sides) without addition of sugar and/or dietary supplements.

In spring 2020 (end of March), a detailed inspection of the colonies was conducted, 40 colonies were selected according to adult bee population, brood areas and food amounts and allotted to 5 groups with 8 colonies in each (Table 1). The activities undertaken after the formation of the groups are shown in Table 2. On March 24, 2020 (Time 0), hygienic behavior was tested by the so called "pin-killed" technique in accordance with the procedure described by Kefuss et al. [84] and modified by Stanimirovic et al. [36]. Briefly, on the one frame per each hive, the diamond area of comb (5 x 6 cm) was marked and all pupae within that area were killed with a pin. The frame was returned to the hive and after 24 hours checked. If more than 95% of the pin-killed cells were cleaned, the colony was considered super-hygienic, if the

efficiency of pupae removal was between 90% and 95%, the colony was proclaimed hygienic, while non-hygienic colonies were those which cleaned less than 90% of the sacrificed brood.

Table 1. Description of groups

Group name	Infection	Supplement
E1-NoV4+Beewell	*N. ceranae* + 4 viruses*	BEEWELL AminoPlus
E2-NoV4	*N. ceranae* + 4 viruses*	–
E3-No	*N. ceranae*	–
E4-V4	4 viruses*	–
C-	Negative control (no infection)	–

*Deformed wing virus (DWV), Acute bee paralysis virus (ABPV), Chronic bee paralysis virus (CBPV) and Sacbrood virus (SBV).

Table 2. Experimental design

Date	Time 0 March 24, 2020	March 27, 2020	Time 1 April 17, 2020	April 17–May 8, 2020	Time 2 August 25, 2020	March 8–22, 2021	Time 3 March 29, 2021
Activities	Assessment of hygienic behavior	Infection	Assessment of hygienic behavior Sampling of bees for laboratory analyses	Supplement application	Assessment of hygienic behavior Sampling of bees for laboratory analyses	Supplement application	Assessment of hygienic behavior Sampling of bees for laboratory analyses

All 40 colonies were super-hygienic (min. 96.69%, max. 98.35% and 97.37% on average). i.e., with the level of behavior expression greater than 95%. Three days after, on March 27, 2020, all 40 colonies were given 2 liters of water-sugar-honey syrup. The syrup was freshly prepared using 300 g meadow honey and 700 g ground sugar, dissolved in 1 liter of water at room temperature and administered. All groups except the negative control (C-) were infected: E1-NoV4+Beewell and E2-NoV4 were given the syrup which contained freshly made macerate of live bees infected with *N. ceranae* and four viruses: (Deformed wing virus - DWV, Acute bee paralysis virus - ABPV, Chronic bee paralysis virus - CBPV and Sacbrood virus – SBV). Group E3-No was infected with *N. ceranae* only, and E4-V4 only with the four viruses. The negative control group (C-) was left uninfected but was given the water-sugar-honey syrup, without the addition of dietary supplements.

On day 21 post infection, on April 17, 2020 (Time 1) hives were tested for hygienic behavior using the pin-killed brood technique [36]. On the same day, bee samples were taken for laboratory analyses and a dietary amino acid and vitamin supplement Beewell AminoPlus (Provet Genome Biotechnology Laboratory, Ankara, Turkey), was

administered to Group E1-NoV4+Beewell, but not to other groups (Table 1). The supplement was applied to the same group two more times in the same quantities in 7-day intervals, according to the instructions of the producer.

In late summer, on August 25, 2020 (Time 2), the colonies were tested again for hygienic behavior, and bees were sampled for laboratory analyses.

From March 8, 2021 the syrup with supplement was administered again (three times in a 7-day interval). On day March 29, 2021 that is 7 days after the third application (Time 3), hygienic behavior was assessed and bees sampled for analyses.

Preparation of the inoculum

The inoculum for the artificial viral infection of the bees was prepared according to de Miranda et al. [85]. From the hives where all the viruses were present (confirmed by PCR) 200 bees (approximately 20 g) were collected from the hive entrance. The bees were macerated and the virus quantity was measured in the suspension. The bees were infected with a volume sufficient to ensure that each bee received the minimum infective dose of 10^6-10^{11} particles [86,87]. For *Nosema* infection, the suspension of *N. ceranae* spores was prepared as described in Fries et al. [88] and added into the syrup to obtain a final concentration of 10^6 spores/ml.

Bee sampling for laboratory analyses for the presence of pathogens

Bees were sampled three times (Time 1, Time 2 and Time 3). Each time, approximately 100 live forager bees were sampled from each colony, directly from the hive entrance after closing it for 20–30 min [89]. Live bees were collected in sterile single-use vessels, immediately stored in dry ice, transported to the laboratory and stored at -20°C until processed.

Detection of Nosema spores and determination of colony level infection

Abdomens of 60 bees from each colony were macerated in 5 ml of water and the suspension was examined microscopically at 400× magnification. In cases of *Nosema*-positive samples, the colony level infection was determined by hemocytometer through the average number of spores per bee in a pooled sample obtained using 60 bees macerated in 60 ml of water (OIE, 2018). The suspensions of all samples were further used for DNA extraction and PCR analyses.

PCR detection and identification of honey bee microsporidian and trypanosomatid parasites

DNA was extracted from 1 mL of sample suspension obtained in the previous step and using DNeasy Plant Mini Extraction Kit (Qiagen, Hilden, Germany) as in Stevanovic et al. [90,91]. For confirmation of *Nosema ceranae* species PCR-RFLP with

nos-16S-fw/rv primers was applied as in Stevanovic et al. [91], while for the detection of *Lotmaria passim* or *Crithidia mellificae*, PCR protocols with primer pairs CmCytb_F/R and LpCytb_F1/R respectively were used as described in Stevanovic et al. [80]. All PCR amplifications were performed in T100™ Thermal Cycler (Bio-Rad, Germany).

RT-PCR detection and identification of honey bee viruses

From each sample, 30 randomly selected bees were crushed and homogenized in a sterile mortar in the presence of 5 ml PBS solution. After homogenisation and centrifugation for 15 min at 5,000×g, 140 µl of supernatant was collected and used for RNA extraction. Total RNA was extracted using ZR Viral RNA Kit™ (Zymo Research, Orange, CA). The average of 2 µg of extracted RNA was used for a single real-time RT-PCR reaction.

Thermal amplifications were performed in Rotor-Gene Q 5plex (Qiagen, Germany) and the presence of DWV, ABPV, CBPV and SBV in bee samples were tested using the Rotor-Gene Probe RT-PCR Kit (Qiagen, Germany), in separate single-step reactions. The primer pairs, probes and thermal protocols were as in our previous work of Cirkovic et al. [92].

Statistical methods

The results for *N. ceranae* infection level (spore counts) and hygienic behavior expression level were tested for normality by using Shapiro–Wilk's test. Given that data for *N. ceranae* spore counts were not normally distributed (Shapiro–Wilk's test, $p<0.05$), log10 transformation was applied, and groups were compared in two-way ANOVA with repeated measures in one factor, followed by Tukey's test. Data for the behavior were compared between the groups over time using one-way ANOVA followed by Tukey's test, and within the group over time using one-way repeated measures ANOVA followed by Tukey's test. Fisher's exact test was used to compare differences in the occurrence of honey bee viruses and *L. passim* between groups. The levels of significance below 0.05 ($p<0.05$) were considered significant. Statistical analysis of the results obtained in the experiment was carried out using statistical software GraphPad Prism version 6 (GraphPad, San Diego, CA, USA).

Ethical approval: The research has been conducted on invertebrates and in compliance with all the relevant national regulations and institutional policies.

RESULTS

Hygienic behavior

The level of expression of hygienic behavior differed significantly ($p<0.05$) between all assessment times (Time 0, Time 1, Time 2 and Time 3) within each group except for the negative control (C-). In all groups that were artificially infected, but not

supplemented (E2-NoV4, E3-No and E4-V4) the level of expression of hygienic behavior consistently decreased through the whole experimental period and was significantly ($p<0.05$) lower in each subsequent assessment time compared to the previous one (Table 3, Figure 1). In group E2-NoV4, all colonies died before the last assessment time, so it was not possible to assess the behavior in Time 3. However, in the supplemented group (E1-NoV4+Beewell), the level of expression of hygienic behavior decreased only before supplement application (from 97.52±0.77 in Time 0 to 87.40±0.86 in Time 1), and after the application of supplement, the behavior consistently increased, being significantly greater in Time 2 (91.11±1.38) compared to Time 1, and in Time 3 (92.98±1.65) compared to both Time 2 and Time 1 (Table 3, Figure 1).

Table 3. Comparison of hygienic behavior between assessment times and between groups

Groups	Assessment time			
	Time 0 (March 24, 2020)	**Time 1 (April 17, 2020)**	**Time 2 (August 25, 2020)**	**Time 3 (March 29, 2021)**
	Mean±SD	**Mean±SD**	**Mean±SD**	**Mean±SD**
E1-NoV4+Beewell	97.52 ± 0.77^{Aab}	87.40 ± 0.86^{Bab}	91.11 ± 1.38^{Ca}	92.98 ± 1.65^{Da}
E2-NoV4	97.42 ± 0.53^{Aab}	86.68 ± 0.82^{Bab}	79.23 ± 1.79^{Cb}	
E3-No	96.69 ± 0.62^{Aa}	88.64 ± 1.30^{Bac}	83.36 ± 0.93^{Cc}	66.28 ± 2.44^{Db}
E4-V4	97.42 ± 0.53^{Aab}	86.05 ± 0.82^{Bb}	81.50 ± 0.88^{Cd}	64.26 ± 1.24^{Db}
C-	97.62 ± 0.69^{Ab}	97.31 ± 0.60^{Ac}	97.21 ± 0.43^{Ae}	96.38 ± 0.43^{Bc}

$^{A, B, C}$ Different superscript capital letters indicate significant differences $P < 0.05$ between assessment times within each group. $^{a, b, c, d}$ Different superscript lowercase letters indicate significant differences $P < 0.05$ between groups within each assessment time. Group names are indicated in Table 1.

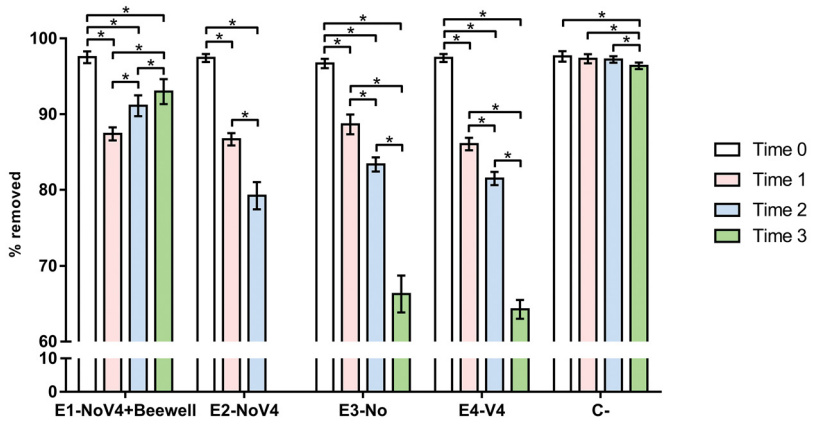

Figure 1. Comparison of hygienic behavior between assessment times within each group. Time 0 – March 24, 2020, Time 1 - April 17, 2020, Time 2 –August 25, 2020, Time 3 –March 29, 2021; *$p<0.05$. Group names are indicated in Table 1.

At the Time 0, hygienic behavior significantly differed ($p<0.05$) only between E3-No and C- groups. At the Time 1, Time 2 and Time 3, hygienic behavior was most expressed in C- group (97.31±0.60, 97.21±0.43 and 96.38±0.43, respectively) and was significantly ($p<0.05$) higher in comparison to all other groups (Table 3, Figure 2). Among the infected groups, hygienic behavior at Time 1 was most expressed in groups E3-No (88.64±1.30) and E1-NoV4+Beewell (87.40±0.86) without significant ($p>0.05$) difference between them, but at Time 2 and Time 3, the group E1-NoV4+Beewell took the lead, reaching the highest level of hygienic behavior (91.11±1.38 and 92.98±1.65, respectively) that was significantly ($p<0.05$) higher compared to all other infected, but not supplemented groups (Table 3, Figure 2).

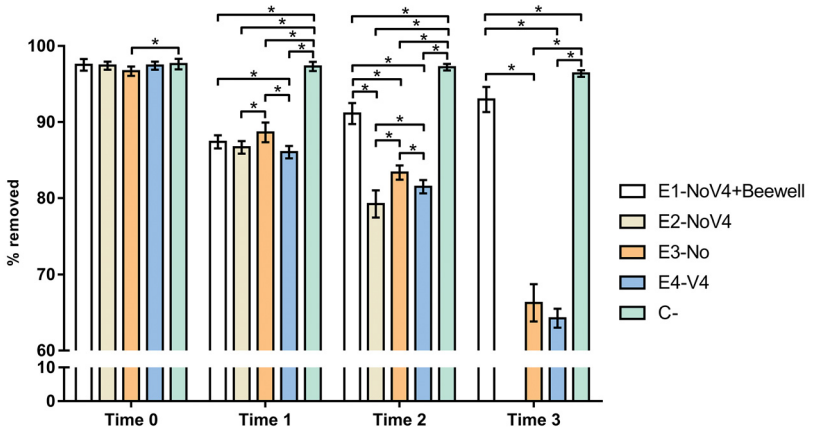

Figure 2. Comparison of hygienic behavior between groups within each assessment time. Time 0 – March 24, 2020, Time 1 - April 17, 2020, Time 2 –August 25, 2020, Time 3 –March 29, 2021; *$p<0.05$. Group names are indicated in Table 1.

Nosema ceranae infection

In all groups, *N. ceranae* infection level (log_{10}) differed significantly ($p<0.05$) between all sampling times (Time 1, Time 2 and Time 3) as shown in Table 4 and Figure 3. Only in supplemented group (E1-NoV4+Beewell), *N. cerane* infection level consistently decreased during the experiment, as was the highest at Time 1, lower at Time 2 and the lowest at Time 3. In non-supplemented groups, either infected (E2-NoV4, E3-No, E4-V4) or not (C-), the situation was reversed: infection level raised through time, reaching the highest value at the last sampling time (Time 3).

At Time 1 and Time 2 (Table 4, Figure 4), the level of *N. ceranae* infection was lowest in the negative control (C-) group and significantly ($p<0.05$) lower compared to all other groups (E1-NoV4+Beewell, E2-NoV4, E3-No and E4-V4). Furthermore, infection level in E4-V4 group was significantly ($p<0.05$) lower than in groups artificially infected with *N. ceranae* spores (E1-NoV4+Beewell, E2-NoV4 and E3-No). However, the infection level in supplemented group (E1-NoV4+Beewell) in Time 2

was significantly ($p<0.05$) lower than in groups artificially infected with *N. ceranae* and not supplemented (E2-NoV4 and E3-No). At Time 3, the lowest infection level was recorded in E1-NoV4+Beewell group and that level was significantly ($p<0.05$) lower than in all other groups, including the negative control (E2-NoV4, E3-No, E4-V4 and C-). The highest *Nosema*-infection level in all sampling times was in groups that were artificially infected with *N. ceranae* but were not supplemented (E2-NoV4 and E3-No); the infection level in those groups was significantly ($p<0.05$) higher compared to both, C- and E4-V4 groups. Between groups E2-NoV4 and E3-No there were no significant differences ($p>0.05$) in *Nosema*-infection level in any of sampling times (Table 4, Figure 4).

Table 4. Comparison of *N. ceranae* infection level (log_{10}) between sampling times and between groups

Groups	N	Sampling times		
		Time 1 (April 17, 2020)	Time 2 (August 25, 2020)	Time 3 (March 29, 2021)
		Mean±SD	Mean±SD	Mean±SD
E1-NoV4+Beewell	8	7.04 ± 0.13^{Aa}	6.82 ± 0.14^{Ba}	6.49 ± 0.20^{Ca}
E2-NoV4	8	7.06 ± 0.06^{Aa}	7.26 ± 0.05^{Bb}	7.45 ± 0.03^{Cb}
E3-No	8	7.18 ± 0.03^{Aa}	7.34 ± 0.07^{Bb}	7.49 ± 0.05^{Cb}
E4-V4	8	5.93 ± 0.08^{Ab}	6.34 ± 0.25^{Bc}	7.25 ± 0.29^{Cc}
C-	8	5.37 ± 0.18^{Ac}	5.51 ± 0.10^{Bd}	6.95 ± 0.05^{Cd}

$^{A, B, C}$ Different superscript capital letters indicate significant differences $p<0.05$ between sampling times within each group. $^{a, b, c, d}$ Different superscript lowercase letters indicate significant differences $p<0.05$ between groups within each sampling time. Group names are indicated in Table 1.

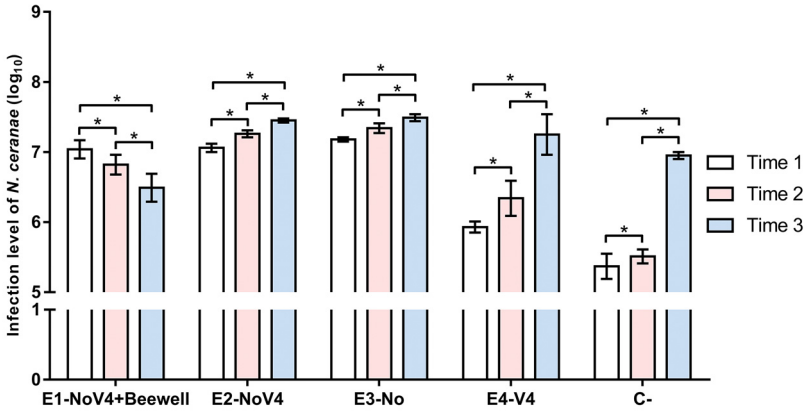

Figure 3. Comparison of *N. ceranae* infection level (log_{10}) between sampling times within each group. Time 1 - April 17, 2020, Time 2 –August 25, 2020, Time 3 –March 29, 2021; *$p<0.05$. Group names are indicated in Table 1.

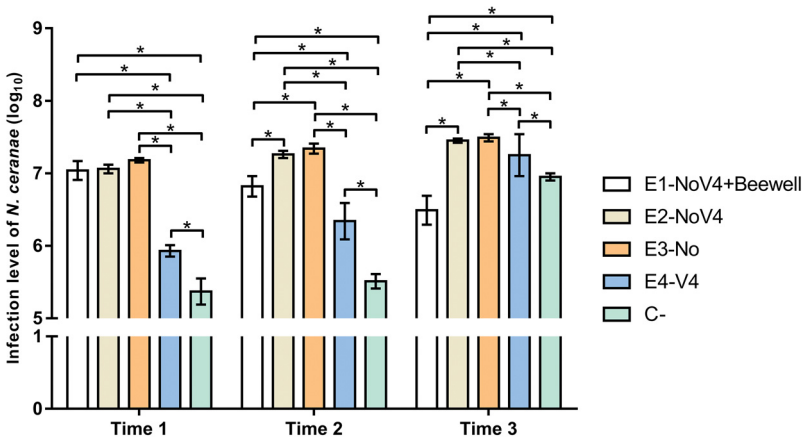

Figure 4. Comparison of *N. ceranae* infection level (log_{10}) between groups within each sampling time. Time 1 - April 17, 2020, Time 2 –August 25, 2020, Time 3 –March 29, 2021; *p<0.05. Group names are indicated in Table 1.

Viruses

DWV: In all groups except in the negative control (C-), there were no significant (p>0.05) differences in the presence of DWV through time, i.e. when the results were compared between sampling times (Time 1, Time 2 and Time 3). In contrast, significant (p<0.05) differences were revealed within C- group when DWV presence was compared between Time 2 and Time 1 and between Time 3 and Time 1 (Figure 5).

At Time 1, hives from artificially infected groups (E1-NoV4+Beewell, E2-NoV4 and E4-V4) were 100% DWV-positive, while groups left uninfected (E3-No i C-) were 100% DWV-negative. At Time 2, C-group became 100% DWV-positive (other groups remained the same status as at Time 1). At Time 3 all five groups were DWV-positive, with all hives (100%) positive in four groups (E1-NoV4+Beewell, E2-NoV4, E4-V4 and C-), and 25% positive hives in E3-No (Figure 5). All differences between groups (within sampling times) were significant (p<0.05).

ABPV: Looking at the presence of ABPV through the time (Figure 5), i.e. between sampling times (Time 1, Time 2 and Time 3), only in E1-NoV4+Beewell group there was a consistent decrease in the percentage of ABPV-positive hives and the difference between Time 1 (100% infected hives) and Time 3 (12.50% infected hives) was significant (p<0.05). In all other groups, there were no significant (p>0.05) differences in percentage of ABPV-positive hives between sampling times.

In each sampling time there were significant differences (p<0.05) in the presence of ABPV (Figure 5): at Time 1, between 100% ABPV-positive groups (E1-NoV4+Beewell, E2-NoV4 and E4-V4) and viruses-free groups (E3-No i C-); at Time 2 the situation changed only in supplemented group (E1-NoV4+Beewell) in a sense of decreasing

the ABPV presence to 50% hives; finally, at Time 3 all five groups were infected, but only E2-NoV4 and E4-V4 remained 100% infected with ABPV, in E1-NoV4+Beewell the percentage of positive hives decreased, and in groups E3-No and C-, the ABPV virus appeared for the first time (12.5% and 37.50% positive hives, respectively). The difference between 100% infected group and any other group was significant ($p<0.05$).

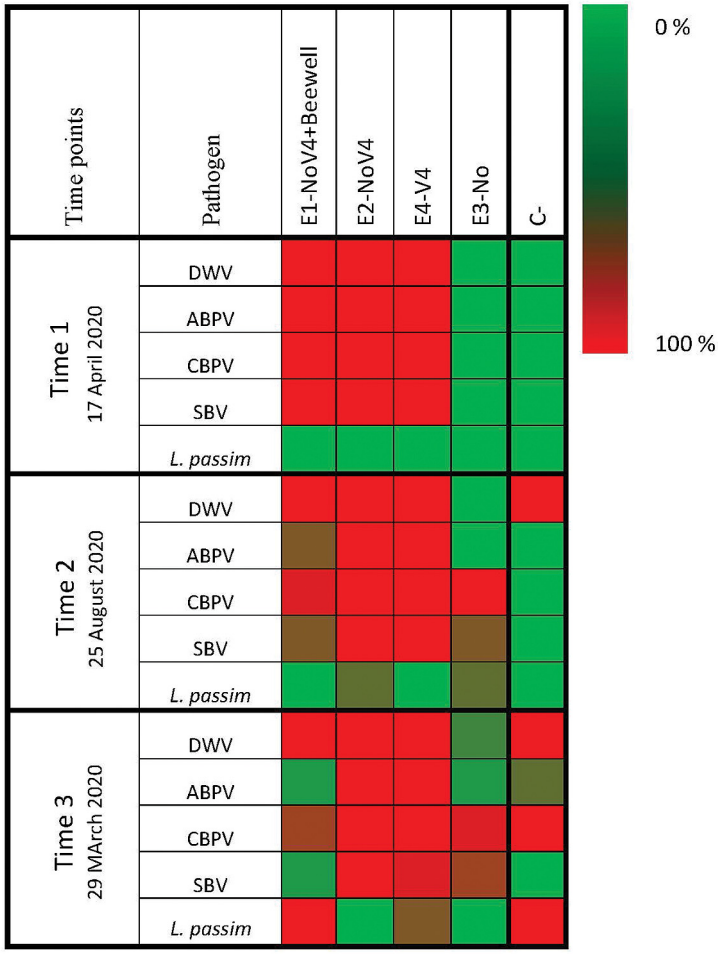

Figure 5. Heatmap of pathogen presence (percentage of positive hives) at different time points in experimental groups. Hives treated with Beewell and infected with *N. ceranae* and four viruses (E1-NoV4+Beewell), hives infected with *N. ceranae* and four viruses (E2-NoV4), hives infected with *N. ceranae* (E3-No), infected with four viruses (E4-V4), negative control (C-). Group names are indicated in Table 1.

CBPV: Comparisons of the CBPV presence in different sampling times (Time 1, Time 2 and Time 3) revealed significant differences only in cases of groups E3-No and C- (Figure 5). In fact, group E3-No was CBPV-free at Time 1, but later became infected

(100% and 87.50% infected hives in Time 2 and Time 3, respectively) and significantly ($p<0.05$) different compared to Time 1. In the C- group, infection with CBPV was registered only at Time 3 (100% infected hives) which was significantly ($p<0.05$) higher compared to Time 1 and Time 2. It is worth to emphasize a consistent decline of CBPV presence in the supplemented group E1-NoV4+Beewell, from 100% infected hives (at Time 1) to 87.50% (at Time 2) and finally to 62.50% (at Time 3).

At Time 1 and Time 2, there were significant ($p<0.05$) differences between CBPV-infected groups and CBPV-free groups (Figure 5).

SBV: All hives in E1-NoV4+Beewell group were SBV-positive at Time 1; 50% of hives remained SBV-infected at Time 2, but only 12.50% at Time 3. The decrease at Time 3 was significant ($p<0.05$) compared to Time 1. In E3-No group, that initially (at Time 1) was viruses-free, 50% of the hives appeared SBV-infected at Time 2 and 62.50% of the hives at Time 3; the last percentage (62.50%) is significantly ($p<0.05$) higher than initial 0% (Figure 5).

Significant ($p<0.05$) differences were affirmed between SBV-positive groups and SBV-free group at Time 1 and Time 2, and also at Time 3 when groups E2-NoV4 and E4-V4 were compared with C- group (that was SBV-free) and supplemented group E1-NoV4+Beewell that contained 12.50% hives infected with SBV (Figure 5).

Trypanosomatids

Only *L. passim* was detected and not *C. mellificae*. Significant ($p>0.05$) differences in presence of *L. passim* were affirmed only in groups E2-NoV4 and E3-No between Time 1 and Time 3 and between Time 2 and Time 3 (Figure 5).

Looking at the sampling times, the presence of *L. passim* was significantly different ($p<0.05$) only at Time 3 in comparisons of 100% infected groups (E2-NoV4 and E3-No) with *L. passim*-negative groups, E1-NoV4+Beewell i C- (Figure 5).

DISCUSSION

Hygienic behavior in honey bees is a complex, disease-resistant, polygenic trait [45,48,49] which genetic basis was investigated by many genomic and tanscriptomic studies [48-55]. The greatest number of candidate genes suggested to contribute to hygienic behavior is 73 as revealed by high-depth full-genome sequencing in a study of Harpur et al. [54]. Moreover, Guarna et al. [45] discovered robust protein expression markers as a completely new tool to select for this behavioral trait.

In this study, we investigated the potential of diet supplementation to enhance the hygienic behavior of honey bee colonies and thus help them fight the most common infections. There were five groups in the experiment: the group that received supplement (E1-NoV4+Beewell) was artificially infected with *N. ceranae* and four viruses (DWV, ABPV, CBPV and SBV); three groups were not supplemented, but were artificially

infected with *N. ceranae* and viruses (E2-NoV4), only with *N. ceranae* (E3-No) or only with viruses (E4-V4); negative control group (C-) was neither supplemented, nor infected.

The results indicate that the tested supplement Beewell AminoPlus significantly stimulates hygienic behaviour. In fact, starting from the day of the supplement application (on 17 April 2020 - Time 1) in E1-NoV4+Beewell group, the hygienic behaviour was significantly better expressed in each subsequent assessment time (Time 2, Time 3) compared to previous one(s), i.e. Time 1, Time 1 and Time 2, respectively. In contrast, in all other (not supplemented) groups (E2-NoV4, E3-No, E4-V4 and C-) the behavior consistently decreased; in the infected groups E2-NoV4, E3-No, E4-V4, it was significantly lower in each subsequent assessment time compared to each previous time point (Figure 1). Hygienic behavior decline in non-supplemented infected groups was much more intensive than its increase in the supplemented group (Table 3, Figure 1) suggesting that it is easier to worsen than to improve this behavior. Nevertheless, only imidacloprid has been reported to significantly impair hygienic activities of worker bees [61], and further studies should investigate the influence of other external factors, both environmental and beekeeping-induced, on hygienic behavior.

The beneficial impact of the Beewell AminoPlus on the hygienic behavior is evident from the results recorded for Time 2 and Time 3 (Figure 2) that indicate the highest level of behavior in the supplemented group and significantly greater that in other infected, but not-supplemented groups. Thus, the supplement stimulated hygienic behaviour of in spite of the negative influence of infections. However, super-hygienic level (>95%) remained only in the negative control group during the entire experiment, while supplemented group reached „hygienic" level (90-95%) in Time 2 and Time 3 but not re-achieved the super-hygienic level.

Nevertheless, this achievement of Beewell AminoPlus is better than other tested supplements. In fact, thymol showed the potential to improve the uncapping and removal of freeze-killed brood but 88% was a maximal removal rate achieved [66], whilst chitosan and peptidoglycan did not alter the hygienic behavior of bees infected with *Nosema* [60].

Changes of the hygienic behavior expression level (consistent decrease) and *N. ceranae* infection level (consistent increase) through time (Figures 1 and 3, respectively) indicate a suppressive effect of infections (induced either by *N. ceranae* only, or by viruses only or by mixed infection of *N. ceranae* and viruses) on hygienic behavior. In contrast, *N. ceranae* infection did not affect hygienic behaviour in study of Valizadeh et al. [60]. This disagreement is probably caused by completely different method applied for behaviour assessment. The most important difference is the assessment time frame: in our study bees were evaluated for hygienic behaviour: 21 day, four months and almost a year following artificial infection (Time 1, Time 2 and Time 3, respectively), while in the

study of Valizadeh et al. [60] bees were only 12-15 days old and even the authors have questioned the impact of *N. ceranae* on bee health in such short period of infection.

In our study, the strongest negative effect of *N. ceranae* and viral infections was recorded in group E2-NoV4 (with mixed artificial infections of *N. ceranae* and four viruses) since all colonies died until the last time point (Time 3). In that group, beside *N. ceranae* and viral infections that were artificially induced, *L. passim* was confirmed, so it could also contribute to the mortality.

Tested supplement showed benefitial effect in terms of control of *N. ceranae* infection; in supplemented group (E1-NoV4+Beewell) *Nosema* spore load significantly and consistently declined through the time, while in all other, non-supplemented groups (E2-NoV4, E3-No, E4-V4 and C-), the situation was inverted (Figure 3). The look on the *Nosema* level data in E1-NoV4+Beewell group in different time points (Figures 3) reveals a great *Nosema*-control effect of applied supplement in wide time-frame after the application, since significant decrease of *N. ceranae* load is evident both after five months of first application set and after 7 days of second application set. Besides, comparison within groups in each time point (Figure 4) revealed that in Time 3 convincingly the lowest *N. ceranae* load was in the supplemented group. Beewell AminoPlus has been already reported to reduce *N. ceranae* spore number in cage experiment, but also to prevent *Nosema*-induced host immunosuppression by modifying the expression of immune-related genes (those that code abaecin, hymenoptaecin, defensin, apidaecin and vitellogenin) in *Nosema*-infected bees [70]. We might assume that in current study the supplement Beewell AminoPlus also potentiated *Nosema* control through enhancing individual bee immunity by up-regulation immune-related genes. Beside those genes that are quite proven to be down-regulated by *Nosema*-parasitism [5,6,30,71,72,93-96] other host genes are also prone to be suppressed by the same factor: genes involved in chitin metabolism and cuticle coatings [95,96], genes related to metabolism of carbohydrates [95,96]; genes encoding odorant binding proteins [96] and genes involved in homeostasis of intestinal tissue, cell apoptosis and renewal [97-99]. We may hypothesize that in the current study Beewell AminoPlus achieved positive effects by stimulation of some of these genes, but further studies are necessary to investigate that. It would be interesting to assess behavioural regulation of the intake of supplement as in de Sousa [100], and the potential on health and reproductive parameters generally affected by supplements [101, 102].

Regarding the viruses (Figure 5), it seems that the supplement did not affect DWV, but contributed to the consistent decrease of ABPV, CBPV and SBV (significant in cases of ABPV and SBV presence between Time 1 and Time 3).

L. passim appeared in all experimentally infected groups, except the supplemented one (Figure 5). In E2-NoV4 and E3-No groups *L. passim* infection was recorded as early as in Time 2 in 37.50% of hives and reaching its presence in 100% of hives in Time 3 that was a significant increase compared to Time 2. In E4-V4 group, *L. passim* was confirmed only at the final time point (Time 3) in 50% of hives. In relation to the

absence of *L. passim* in E1-NoV4+Beewell group, we may assume that the supplement was the one that prevented the invasion of that parasite.

CONCLUSION

The impact of diet supplementation on hygienic behavior of honey bee colonies has been poorly known. The supplement tested in this study expressed a positive influence on hygienic behaviour of colonies infected with *N. ceranae* and four viruses. In fact, starting from the day of the supplement application, the hygienic behaviour was significantly better expressed in each subsequent assessment time compared to previous one(s), contrary to non-supplemented groups, either infected or not. The supplement also helped colonies in terms of control of *N. ceranae* infection. Further studies are needed to investigate if the supplement has potential to prevent *L. passim* infection.

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Authors' contributions

ZS conceived and designed the study and coordinated experiment performance, participated in writing and reviewing of the manuscript. UG and MR carried out field experiment; SJ and MN carried out laboratory analyses; BV performed the statistical analysis; ZS, BV, JS, UG and MR made substantial contributions to interpretation of data. JS wrote the manuscript with imput from all authors. All authors discussed the results, read and approved the final version..

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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DIJETETSKI SUPLEMENT PODSTICANJEM HIGIJENSKOG PONAŠANJA POMAŽE PČELINJIM DRUŠTVIMA U BORBI SA INFEKCIJAMA

Zoran STANIMIROVIĆ, Uroš GLAVINIĆ, Marko RISTANIĆ, Stefan JELISIĆ, Branislav VEJNOVIĆ, Mia NIKETIĆ, Jevrosima STEVANOVIĆ

Higijensko ponašanje pčela je složena poligena osobina i predstavlja prirodni mehanizam biološke odbrane protiv virusnih, bakterijskih, gljivičnih i infekcija legla protozoama, ali i protiv grinje *Varroa destructor,* koja infestira pčelinje leglo i adultne insekte. Cilj istraživanja je bio procena efekata dijetetskog aminokiselinsko-vitaminskog suplemenata "BEEWELL AminoPlus" na higijensko ponašanje pčelinjih zajednica vrste *Apis mellifera* u borbi protiv mikrosporidijalnih i virusnih infekcija. Jednogodišnji eksperiment je sproveden na 40 društava raspoređenih u pet grupa: jedna suplementirana i inficirana sa *Nosema ceranae* i četiri virusa (virus deformisanih krila - DWV, virus akutne paralize pčela - ABPV, virus hronične paralize pčela - CBPV i virus mešinastog legla – SBV), tri hranjene bez dodatka suplementa, ali inficirane sa *N. ceranae* i/ili virusima i jedna negativna kontrolna grupa. Pored navedenih patogena, tripanozome pčela su takođe praćene u svim grupama.

Primenjeni suplement "BEEWELL AminoPlus" uslovio je konzistentno značajano povećanje stepena higijenskog ponašanja uprkos negativnom dejstvu *N. ceranae* i virusnih infekcija. *N. ceranae* i virusi su dosledno i značajno vodili smanjenju nivoa higijenskog ponašanja pčela, ugrožavajući, život i opstanak pčelinjih zajednica. Testirani suplement je pokazao antinozematozni efekat, jer je nivo infekcije *N. ceranae* značajno i konzistentno opadao samo u grupi tretiranoj ispitivanim suplementom. Među zaraženim grupama uočili smo da je samo ona grupa koja je u prihrani dobijala suplement bila je slobodna od tripanozomalne infekcije vrstom *Lotmaria passum*. Može se zaključiti da primenjen suplement poboljšava higijensko ponašanje pčelinjih zajednica i pomaže im u borbi protiv najčešćih infekcija.