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# Effect of feed supplementation with probiotics and postbiotics on strength and health status of honey bee (*Apis mellifera*) hives during late spring

Eduardo José García-Vicente [a,b,*], María Martín [b], Ismael Rey-Casero [b], Ana Pérez [a], Remigio Martínez [c], María Bravo [d], Juan Manuel Alonso [c], David Risco [a]

[a] Department of Animal Medicine, Facultad de Veterinaria, Universidad de Extremadura, Av. de la Universidad s/n, 10003 Cáceres, Spain
[b] Neobéitar S.L. Av. Alemania 6 1°B, 10001 Cáceres, Spain
[c] Department of Animal Health, Facultad de Veterinaria, Universidad de Extremadura, Av. de la Universidad s/n, 10003 Cáceres, Spain
[d] Ingulados, Miguel Servet 11-13, 10004 Cáceres, Spain

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ABSTRACT

Currently, beekeeping faces many risks, such as deteriorating health of honeybees in hives, which results in high mortality rates, mainly during winter. An important consequence is the emergence/re-emergence of communicable diseases such as varroosis or nosemosis. These diseases jeopardize the continuity of the sector because of the absence of effective treatments and harmful residues that they can be retained on wax or honey. This study aimed to evaluate how feed supplementation with probiotic and postbiotic products derived from lactic acid bacteria affected the strength, dynamic population, and sanitary parameters of honey bees. Three groups of 30 hives were established and fed with feed supplemented with control, probiotic, or postbiotic products, with a total of nine applications over two months in late spring. Two monitoring tests were conducted to evaluate the strength and health status of hives. Hives that consumed postbiotic products enhanced their strength, increased bee population and egg laying of the queen, and maintained their reserves of pollen, whereas these parameters decreased in hives belonging to other groups. Furthermore, although the results suggested a favorable effect of postbiotic products on the trend of *N. ceranae* infection levels, probiotics showed intermediate results. While awaiting long-term results regarding *V. destructor* infestation, which showed similar trends in all groups, feed supplementation with postbiotics could be an important tool for beekeepers to enhance the strength and health status of their hives.

## 1. Introduction

Honeybees (*Apis mellifera*) are social insects that play an essential role in the ecosystem. It is one of the most important pollinators and substantially influence agriculture and biodiversity (Abrol, 2012). The insects, particularly *A. mellifera*, pollinate approximately 84% of all commercial species of crops, thereby influencing approximately one-third of global food production (Free, 1993; Richards, 2001; Williams, 2002).

Honey bees have been used for >40,000 years via beekeeping practices to provide highly valued food, honey, and other products such as pollen, royal jelly, wax, and propolis. Thus, beekeeping is an important economic and developmental resource in many regions of the world (Chirsanova et al., 2021).

However, beekeeping faces numerous risks that are jeopardizing its continuity. Climate change or uncontrolled use of pesticides in agriculture are some factors causing severe consequences on beekeeping, decreasing production, and inducing high mortalities of hives (Halm et al., 2006; Salehizadeh et al., 2020; Samson-Robert et al., 2014).

Other factors that negatively influence hives include emerging or re-emerging pathogens such as *Nosema ceranae* and *Varroa destructor*. *N. ceranae* is a fungus that affects the honeybee digestive system, causing intestinal damage, immunosuppression, and reduced survival (Botías et al., 2012; Paris et al., 2018). Traditionally, it has been treated with fumagilin, an antibiotic with highly variable effectiveness depending on the season and apiary location (Huang et al., 2013). Moreover, it can be

* Corresponding author at: Department of Animal Medicine, Facultad de Veterinaria, Universidad de Extremadura, Av. de la Universidad s/n, 10003 Cáceres, Spain.
*E-mail addresses:* edgarciav@alumnos.unex.es (E.J. García-Vicente), maria@ingulados.com (M. Bravo), remimar@unex.es, jmalonso@unex.es (J.M. Alonso), riscope@unex.es (D. Risco).

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toxic to humans, favor the generation of bacterial strains with antibiotic resistance, and leave residues on honey or other products (van den Heever et al., 2014).

Alternatively, *V. destructor* is a mite whose origin is located in Asia, as an *Apis cerana* parasite. It infected *A. mellifera* during the first half of the 20th century and spread rapidly worldwide. Currently, most hives are infested with *V. destructor* (Reams and Rangel, 2022; Rosenkranz et al., 2010; Traynor et al., 2020). Mite infestation causes several injuries to honey bee colonies, such as malformations, weight loss, and weakness (Giacobino et al., 2016); however, it can also infect or transmit many bee viruses, such as deformed wing virus (DWV) or chronic bee paralysis virus (CBPV), and act synergistically with them (Mondet et al., 2014). Moreover, mites can induce changes in the honeybee bacteriome (Hubert et al., 2017).

Amitraz or fluvalinate are used for varroosis control; however, their effectiveness is highly variable due to resistance or tolerance on the mites owing to extensive use (Rinkevich, 2020). Moreover, these substances could leave residues on wax or honey (Korta et al., 2001).

Thus, beekeeping must find new alternatives and natural resources that can help control these pathogens without residues in their products and enhance the strength of the hives. A novel approach involves the use of bioactive compounds, such as probiotics or postbiotics, obtained from the intestinal microbiota of bees (Saccà and Lodesani, 2020; Tejerina et al., 2020).

The microbiota could be an important source of beneficial microorganisms, mainly lactic acid bacteria, which can enhance host defense mechanisms, as they directly affect pathogens via the production of antimicrobial molecules and their ability to interact with the immune system (Bravo, 2021; Pachla et al., 2021; Wang et al., 2020).

Bioactive compounds can improve growth, production rates, and general status in numerous species, in addition to preventing and controlling many diseases and their associated injuries and symptomatology (Bajagai, 2016; Bravo, 2021; Pachla et al., 2021).

Probiotic compounds derived from bacteria like *Ligilactobacillus salivarius* reduce the incidence of *V. destructor* and *N. ceranae* in commercial apiaries when it is added to supplementary feed (Tejerina et al., 2020). However, no studies have focused on evaluating the effects of postbiotic products (metabolites/cell-free supernatants and soluble factors secreted by live bacteria (Barros et al., 2020)) derived from similar bacteria used as supplementary feed. Only laboratory experimental tests have been performed, exploring the effects of postbiotics on the sanitary and nutritional parameters of honeybees (De Piano et al., 2020; Saccà and Lodesani, 2020).

The aim of this study was to evaluate the effects of feed supplementation with two different bioactive compounds (probiotics and postbiotics) on the sanitary parameters (*V. destructor*, *N. ceranae*, DWV, and CBPV) and productivity of honey bees under field conditions.

## 2. Material and methods

### 2.1. Elaboration of supplementary products

Three different supplementary products were used to feed the hives: probiotics, postbiotics, and control supplements. All were prepared based on a solution of sucrose and water (125 g/l), elaborated, and applied in the hives.

The probiotic was elaborated with a solution of three lactic acid bacteria of the *Lactobacillus* genus, which were selected based on the results of previous studies that showed immunomodulatory capabilities in other species (Bravo et al., 2022). These bacteria were inoculated on 100 ml of liquid growth medium MRS Scharlau© and incubated for approximately 48 h, until a concentration of $10^8$ CFU/ml was attained. Finally, one ml of the culture was added to 999 ml of the glucose solution to attain a concentration of $10^5$ CFU/ml.

The postbiotic was prepared using the same culture of lactic acid bacteria as was used to obtain the probiotic product at the same concentration. However, in this case, after the 48 h incubation period, the liquid growth medium was inactivated by heat at 80 °C for one hour. Following this, the absence of live bacteria was studied by culturing them in the MRS medium. One ml of this postbiotic product was added to 999 ml of the glucose solution to feed the hives.

Finally, to elaborate the supplementary feed for the control group, one ml of sterile MRS medium was added to 999 ml of glucose solution.

### 2.2. Experimental design

Three groups of 30 hives with different supplementation patterns were generated: probiotic (PRO), postbiotic (POS), and control (C). Ten hives from each group were located at three different locations, all in traditional beekeeping regions in Extremadura, Spain. These areas, with a typical Mediterranean climate characterized by gentle winters and hot summers with little rainfall (Sancho, 2019), have diverse flora, including *Quercus ilex* or *Quercus suber*, *Eucaliptus camaldulensis* and *Eucaliptus maidenii*; species of *Pinus* genus; shrubs such as rockrose (*Cistus ladanifer*), rosemary (*Salvia rosmarinus*), or broom (*Cytisus scoparius*); and grasslands.

The studied hives received a total of nine supplementations, which were scheduled weekly for two months (25th of May 2021 to 20th of July 2021), and comprised the application of one litre of control, probiotic, or postbiotic supplement (according to the group) on vertical troughs that were previously installed in each hive. The amount of food consumed was recorded one week after each supplementation.

### 2.3. Monitoring of beehives

The strength parameters of the beehives were monitored twice: at the beginning of the experiment (M1), just before the application of supplementary feed (25th of May), and at the end of the experiment (M2), one week after the last supplementation (27th of July).

Thus, the number of adult bees, number of capped and opened broods, and honey/pollen reserves were estimated for each hive in M1 and M2, as was described by Delaplane et al. (2013). Briefly, the number of bees per colony was estimated by calculating the percentage of the surface occupied on both sides of each frame. Similarly, the remaining parameters were referred to as the percentage of the surface occupied by each parameter. To reduce bias, these parameters were estimated in duplicate by two different technicians, and the mean value was calculated. The estimates were carried out at the same hour, to avoid a greater variability in the number of bees outside the hive.

### 2.4. Diseases diagnosis

Using hive monitoring, approximately 400 adult bees from each hive were sampled for diagnostic purposes, allowing the assessment of both initial and final infestation levels of *V. destructor*, *N. ceranae*, DWV, and CBPV. Sampled bees were collected at extreme frames to avoid damaging queen bees or broods in the middle frames of the hive and were taken to the laboratory under refrigeration. Once in the lab, about 30 bees were preserved at −20 °C to check *N. ceranae*, whereas other 30 bees were preserved at −80 °C for checking viral load. The rest of the sampled bees were refrigerated to check for *V. destructor*.

To estimate *V. destructor* infestation levels, about 300 bees were immersed in a 5% ethanol solution and were shaken for five minutes, following which they were passed through a sieve, during which the total numbers of bees and mites were counted (Calatayud and Verdú, 1992). Following this method, the number of phoretic *V. destructor* in each hive was determined and expressed as the number of mites per 100 bees. Finally, hives were categorized depending on the intensity of *V. destructor* infestation at three different levels: “0” or no presence of mites on sample of bees; “1” or low level of infestation when less than three mites were found per hundred bees (<3%); and “2” or high level of infestation when more than three mites were found per hundred bees

(>3%) (Giacobino et al., 2017).

To assess the presence of *N. ceranae*, digestive tracks were extracted from 30 bees per hive. The guts were brought in five ml of sterile DNase-free water and homogenized in a stomacher bag for five minutes. Subsequently, one ml of each sample was incubated with 200 µl of germination solution (NaCl 0.5 M + $NaCHO_3$ 0.5 M) at 37 °C for 15 min. This final solution was used as matrix to carry out a DNA extraction using the NukEx Complete Mag RNA/DNA® (Gerbion GmbH & Co. KG, Germany) using a KingFisher™ Flex, Thermo Fisher™ Scientific Inc. The DNA concentration of each sample was measured using a NanoDrop™ 2000 (Thermo Fisher™ Scientific Inc.), and quantitative PCR was performed, as was previously described (Bourgeois et al., 2010) (See Table 1). Briefly, for each reaction, 10 µl of Premix Ex Taq™ (2×) (TaKaRa Bio, Japan), 0.2 µM of each primer and ROX Reference Dye, 0.8 µl of Probe, and 2 µl of DNA sample (5 ng of DNA) were taken in a total volume of 20 µl PCR reaction mixture. Cycling parameters of PCR consisted of an initial denaturation at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, and 60 °C for 34 s. Duplicate reactions were performed for template samples, standards, and non-template controls. The number of DNA copies present in each sample was estimated based on a standard curve calculated using thermocycler-specific software (Applied Biosystems 7300, Thermo Fisher Scientific Inc., USA) and expressed as the number of copies of *N. ceranae* DNA per ng of total DNA.

To assess the presence of DWV and CBPV, 20 bees per hive were brought in five ml of sterile PBS and homogenized for five minutes in a Blender Smasher™ (BioMérieux, Spain). RNA extraction was performed from one milliliter of homogenized mix per sample using the NukEx Complete Mag RNA/DNA® (Gerbion GmbH & Co. KG, Germany) and KingFisher™ Flex (Thermo Fisher™ Scientific Inc.). Subsequently, retrotranscription of samples was carried out to obtain cDNA using the PrimeScript™ RT Reagent Kit (TaKaRa, Japan) following the manufacturer's recommendations. Total RNA was quantified using NanoDrop™ 2000 (Thermo Fisher™ Scientific Inc.). qPCR for DWV and CBPV was performed using Premix Ex Taq™ (TaKaRa Bio, Japan), as was previously described (Schurr et al., 2019) (See Table 1). The results are expressed as the number of copies of viral cDNA per ng of total DNA from each sample.

### 2.5. Data analysis

The general status (number of bees, brood, pollen, and honey) and sanitary parameters (infestation levels of *V. destructor*, *N. ceranae*, DWV, and CBPV) were compared between the experimental groups at both the initial (M1) and final (M2) time points. Parametric statistical tests (one-way ANOVA (F)) were used when the variables showed a normal distribution. Otherwise, nonparametric statistical tests (Kruskal-Wallis test (H)) were performed, followed by post-hoc tests (Tukey's HSD test or pairwise Wilcoxon test).

Furthermore, the existence of intragroup differences between M1 and M2 was explored using parametric or nonparametric statistical tests depending on the normality of the variables (paired T-student (T) *t*-test or Wilcoxon test (V)).

Hives that consumed <80% of supplementary products applied were excluded from statistical analysis. Statistical analysis were performed using R v4.1.2 software. *P* values <0.05 were considered statistically significant.

**Table 1**
Primers used on q-PCR for DNA/cDNA quantification of Nosema ceranae, Deformed Wing Virus (DWV) and Chronic Bee Paralysis Virus (CBPV).

| Species | Primers | Reference |
|---|---|---|
| *N. ceranae* | Forward 5′-AAGAGTGAGACCTATCAGCTAGTTG-3′<br>Reverse 5′-ATCTCTCATCCCAAGAGCATTGC-3′<br>Probe 5′-ACTTACCATGCCAGCAGCCAGAAGA-3′ | Bourgeois et al., 2010 |
| DWV | Forward 5′-GCGGCTAAGATTGTAAATTG-3′<br>Reverse 5′-GTGACTAGCATAACCATGATTA-3′<br>Probe 5′-CCTTGACCAGTAGACACAGCATC-3′ | Schurr et al., 2019 |
| CBPV | Forward 5′-CGCAAGTACGCCTTGATAAAGAAC-3′<br>Reverse 5′-ACTACTAGAAACTCGTCGCTTCG-3′<br>Probe 5′-TCAAGAACGAGACCACCGCCAAGTTC-3′ | Schurr et al., 2019 |

## 3. Results

### 3.1. General parameters

The mean consumption registered throughout the experiment was 750 ml on C, 863.3 ml on POS, and 825.7 ml on PRO (see supplementary file 1); no significant differences were detected among the different products supplied.

The mean values obtained for the general parameters measured at M1 and M2 are summarized in Table 2. Briefly, the mean value of the total number of adult bees was significantly lower in POS than that in the rest of the groups at M1 (H = 20.21; $p$ = 4.095E-5). Similarly, pollen reserves were significantly lower in POS than that in C at M1 (H = 9.29, $p$ = 0.01). However, no differences were detected in these parameters among the groups at M2 (Fig. 1).

The number of adult bees (V = 75.5; $p$ = 0.035) and opened broods (V = 35; $p$ = 0.001) significantly increased throughout the POS experience, whereas the number of capped broods ($T$ = 2.96; $p$ = 0.007) decreased in this group.

Regarding feed reserves, all groups increased their honey reserves during the experiment (C: V = 1, p = 0.001; POS: $T$ = −9.45, $p$ = 2.181E-9; PRO: $T$ = −7. 71; $p$-value = 2.897E-7). Pollen reserves remained stable in POS and PRO, whereas a significant decrease was observed in C between M1 and M2 ($T$ = 2.33, $p$ = 0.033).

### 3.2. Diseases diagnosis

Table 3 shows the average infestation levels of *V. destructor*, *N. ceranae*, DWV, and CBPV registered in all groups at M1 and M2.

Briefly, no significant differences were observed in pathogen diagnosis among the groups at M1. However, lower *N. ceranae* infestation levels were detected at M2 in POS than those in C (H = 6.4; $p$ = 0.041) (Fig. 2).

Regarding the differences in pathogen diagnosis between M1 and M2 in each group, POS (V = 7; p = 0.001) and PRO (V = 0; $p$ = 0.003) significantly increased the intensity of infestation by *V. destructor*. In addition, PRO significantly increased the DWV load between M1 and M2 (V = 33; $p$ = 0.006). Finally, no significant differences were detected in the CBPV loads among the groups or monitoring.

## 4. Discussion

The results obtained in this study showed that supplementation with postbiotic compounds in late spring improved key parameters related to the general strength and health status of the hives.

First, hives supplemented with postbiotic products were the only ones that showed an increase in their population of adult bees, despite starting the experiment with significantly fewer bees than the other groups. The number of adult bees in a hive is one of the best parameters to determine their strength and vigor, and it is described as the best factor to predict the surveillance of the colony the following winter (Harris, 2010; Lee and Winston, 1987). The increase in bee population could be due to the higher number of broods during the experiment. The number of opened broods was significantly higher in the POS between M1 and M2.

Late spring, when the experiment began, is the time of the year when beehives reach their maximum development in the region where they were located. After this period, the population remains stable or

**Table 2**
General parameters measured at the beginning (M1) and at the end (M2) of the experience by groups. All parameters but "bee estimation" are expressed as $dm^2$ of frames occupied by each one. *P*-values <0.05 were considered statistically significant. Values marked with an "a" and a "b" showed significant differences in mean comparison among the groups in the same monitoring and between M1 and M2 on the same group respectively.

| | Bee estimation | | Capped brood | | Opened brood | | Honey | | Pollen | |
|---|---|---|---|---|---|---|---|---|---|---|
| | M1 | M2 | M1 | M2 | M1 | M2 | M1 | M2 | M1 | M2 |
| **C (*n* = 17)** | 11,615 [a] | 9886 | 30.61 | 27.3 | 12.54 | 16.71 | 40.7 [b] | 94.16 [b] | 27.18 [a b] | 18.68 [b] |
| **POS (*n* = 24)** | 6258 [a b] | 8295 [b] | 32.24 [b] | 25.27 [b] | 8.16 [b] | 21.46 [b] | 46.35 [b] | 84.18 [b] | 18.13 [a] | 18.31 |
| **PRO (*n* = 20)** | 10,543 [a] | 9088 | 29.37 | 25.28 | 11.21 | 15.36 | 55.48 [b] | 87.6 [b] | 21.18 | 20.48 |

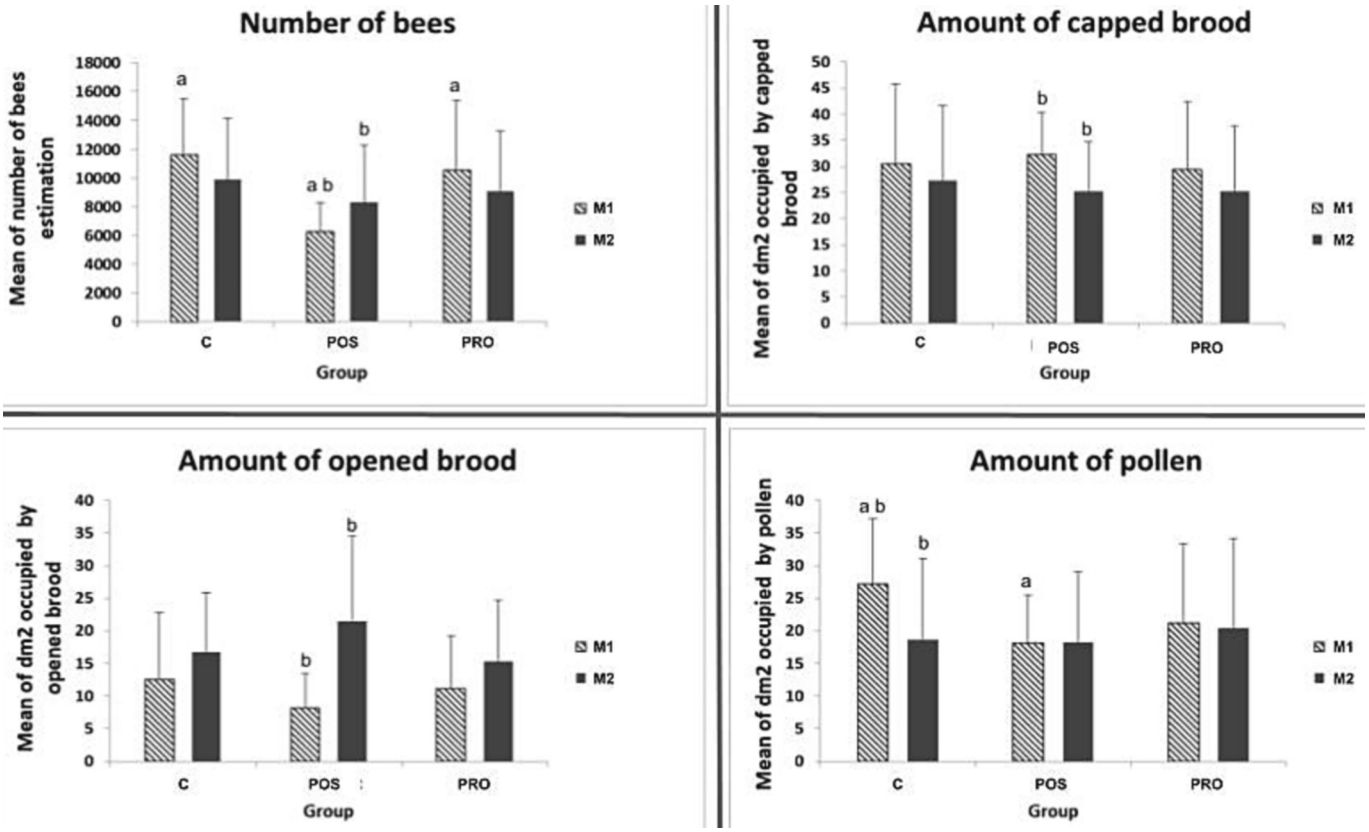


**Fig. 1.** Left to right and top to bottom: mean of bee estimation for each group and monitoring; mean of dm2 occupied by capped brood; mean of dm2 occupied by opened brood; and mean of dm2 occupied by pollen reserves. P-values <0.05 were considered statistically significant. *P*-values <0.05 were considered statistically significant. Values marked with an "a" and a "b" showed significant differences in mean comparison among the groups on the same monitoring and between M1 and M2 on the same group respectively.

**Table 3**
Sanitary parameters measured at the beginning (M1) and at the end (M2) of the experience by groups. The *Varroa destructor* intensity of infestation and the DNA loads of Nosema ceranae, Deformed Wing Virus (DWV) and Chronic Bee Paralysis Virus (CBPV) (expressed as number of DNA copies of each one per one ng of total DNA of sample) for each study group or monitoring are shown. P-values <0.05 were considered statistically significant. Values marked with an "a" and a "b" showed significant differences in mean comparison among the groups on the same monitoring and between M1 and M2 on the same group respectively.

| | *V. destructor* | | *N. ceranae* | | DWV | | CBPV | |
|---|---|---|---|---|---|---|---|---|
| | M1 | M2 | M1 | M2 | M1 | M2 | M1 | M2 |
| **C (n = 17)** | 0.18 | 0.47 | 1.71E+3 | 3.39E+3 [a] | 7.18E+4 | 1.58E+5 | 0.02 | 0.02 |
| **POS (n = 24)** | 0.08 [b] | 0.86 [b] | 2.5E+3 | 1.2E+3 [a] | 7.9E+4 | 2.53E+5 | 6.66 | 0.05 |
| **PRO (n = 20)** | 0.25 [b] | 0.95 [b] | 1.73E+3 | 2.08E+3 | 1.12E+5 [b] | 3.79E+5 [b] | 0.02 | 0.26 |

decreases (Hatjina et al., 2014). This situation was also observed in C; however, POS increased the populations of bees and broods. This increase in dynamic population has been previously documented when hives were supplemented with probiotics and postbiotics (De Piano et al., 2017; Sabaté et al., 2012); however, in this case, only POS showed this effect.

Hive reserves strongly depend on the weather of the year and food resources (Huang and Robinson, 1996). All groups increased their honey reserves significantly during the experiment, indicating that nectar resources were available in the surroundings of the apiary. However, although C began the experience with significantly more pollen reserves than that in the other groups, these reserves decreased significantly during the experiment, indicating a lack of pollen resources during this period, whereas the other groups maintained similar values. The POS

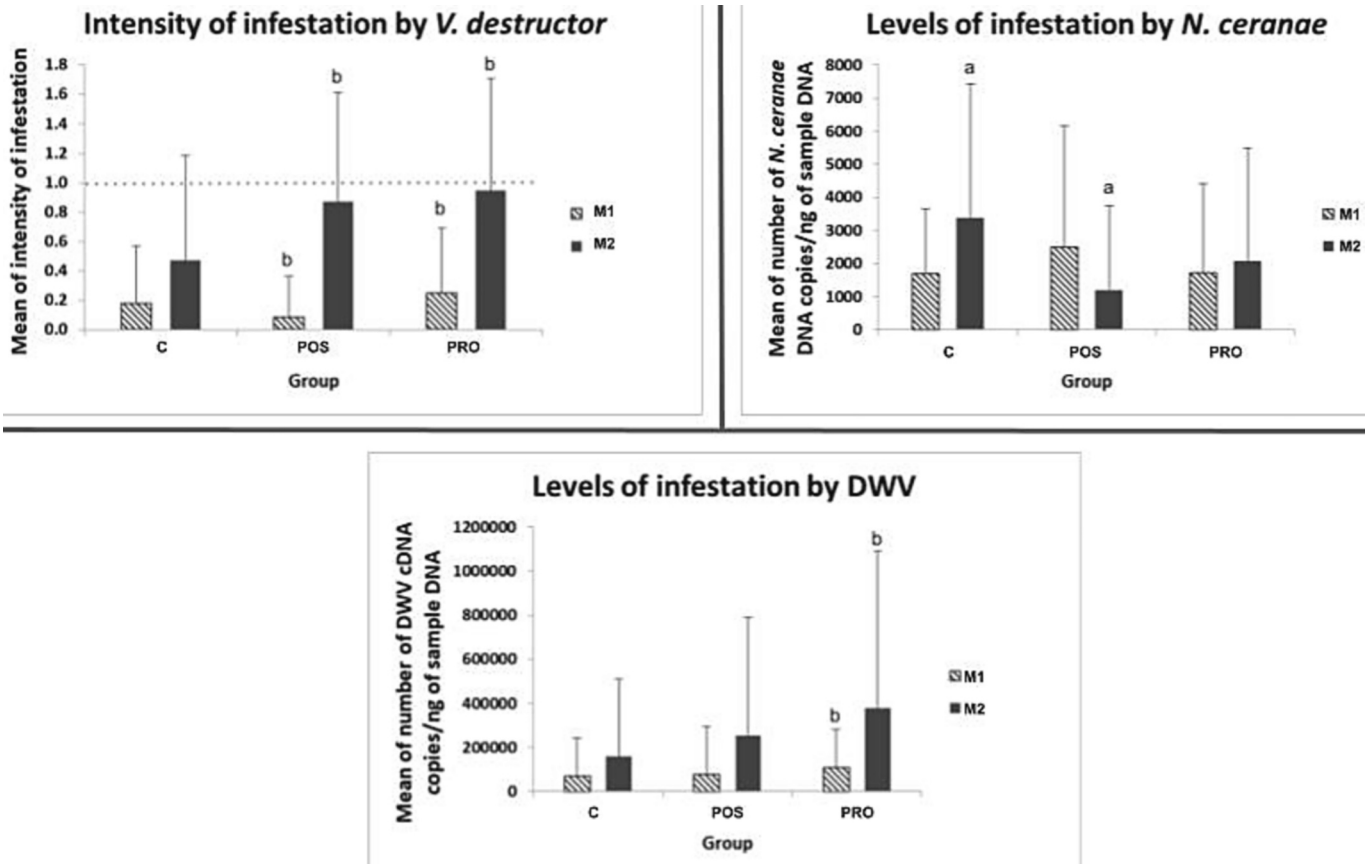


**Fig. 2.** Left to right and top to bottom: intensity of infestation by *V. destructor*, and load of N. ceranae and DWV (expressed as number of DNA copies of each one per one ng of total DNA of sample) for each study group and monitoring. P-values <0.05 were considered statistically significant. Values marked with an "a" and a "b" showed significant differences in mean comparison among the groups on the same monitoring and between M1 and M2 on the same group respectively.

case must be specifically mentioned, because this group had more opened broods, which indicates higher requirement for pollen protein (Brodschneider and Crailsheim, 2010), and even so, its reserves remained stable. This could be because of the higher number of foreign bees in this group, which collected large quantities of pollen from the field.

Among the studied pathogens, the infection levels of *N. ceranae* were lower in POS than those in C at the end of the experiment. These results suggest that supplementation with the postbiotic product contributed to a decrease in the load of *N. ceranae*. This was previously described when feed supplementation with postbiotic products derived from the culture of *Lactobacillus johnsonii* in beehives decreased the load of *N. ceranae* in treated bees (De Piano et al., 2017). However, probiotic supplementation did not change the levels of infestation by *N. ceranae*, which was expected based on other studies (Tejerina et al., 2020; Tlak Gajger et al., 2020).

The infestation levels of *V. destructor* did not differ significantly among the groups at the beginning or end of the experiment. All groups showed a positive trend in mite infestation, but maintained low levels. This increase was significant for both PRO and POS, which may be due, at least in the latter case, to an increase in its brood. The life cycle of the mite is closely related to that of bees, and it uses cells with broods to breed inside them, feeding on larvae. Thus, a larger number of bee broods provides more opportunities for mites to breed and increase their populations (Rosenkranz et al., 2010).

These results differ from those of other studies that demonstrated the effectiveness of feed supplementation with probiotics derived from lactic acid bacteria against mites (Tejerina et al., 2020), whereas no studies had explored the effect of postbiotics as a feed supplement against *V. destructor* under field conditions. These differences may be due to the different temporalities of the studies, which observed the effect over a period of some years, for which new studies were proposed with longer durations to check the possible long-term effects of postbiotics and probiotics on hives.

Finally, the DWV load was not significantly different among the groups, and positive trends through experience were similar in all groups, although it was significant only in PRO. Similarly, CBPV load did not differ among the groups.

To the best of our knowledge, this is the first report of bee hives supplemented with postbiotics under field conditions. Improvements were registered in population dynamics (increasing the number of adult bees and egg laying of the queen), pollen reserves, and health status (low levels of *N. ceranae*). The effects of postbiotic supplementation were greater than those of the probiotic products obtained from the same strains (maintenance of pollen reserves). Probiotic supplementation has been previously associated with improvements in parameters, such as the number of bees, honey yield, and *V. destructor* infestation (Alberoni et al., 2018; Sabaté et al., 2012; Tejerina et al., 2020). The lack of an effect of probiotics in this study might be related to the low surveillance of live bacteria under field conditions or minimal resistance to climatic characteristics (Coghetto et al., 2016; Lacroix and Yildirim, 2007); however, this parameter was not recorded.

Thus, postbiotic supplements derived from lactic acid bacteria could be an interesting tool for enhancing the strength and health status of beehives. Nevertheless, further research evaluating reliable supplementation protocols over a longer period is necessary to determine the real potential of these products for beekeeping.

## Declaration of Competing Interest

None.

## Acknowledgements

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.rvsc.2023.05.001.

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