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Research Article

Protective role of plant-based pollen substitute diets against *Nosema* spores in *Apis mellifera* colonies

Ashish R. Gawali*
Department of Zoology, Dr. Babasaheb Ambedkar Marathwada University, Chhatrapati Sambhajinagar (Aurangabad)- 431004 (MS), India
Bhalchandra B. Waykar
Department of Zoology, Dr. Babasaheb Ambedkar Marathwada University, Chhatrapati Sambhajinagar (Aurangabad)- 431004 (MS), India

*Corresponding author. Email: ashishgawali8888@gmail.com

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Abstract
Nosema sp. causes a significant threat to honeybee populations, making it crucial to find effective mitigation strategies. The present study examines the impact of plant-based pollen substitute diets on *Nosema* spore infection in *Apis mellifera* L. colonies. The study explores the potential of phytochemicals, which have antimicrobial properties, as natural treatments to reduce *Nosema* infection. Seven experimental groups (Diet-2 to Diet-8 along with Diet-1 (control) of colonies were fed with different pollen substitute diets containing medicinal plant leaves such as coriander (*Coriandrum sativum*), moringa (*Moringa oleifera*), tulsi (*Ocimum sanctum*) and lemongrass (*Cymbopogon citratus*); fruit powders of amla (*Embilica officinalis*), guava (*Psidium guava*) and mixed diet containing equal quantities of all plant leaves and fruit powders, along with common ingredients like defatted soya flour, skimmed milk powder, brewer's yeast, turmeric, vitamins and sugar syrup. The Diet-2 to Diet-8 were compared to a control group of colonies (Diet-1), which was only fed with sugar syrup. *Nosema* spores were counted in samples of 40 worker bees from experimental and control groups of colonies before feeding and at intervals of 8, 16, 24 and 32 days using an Improved Neubauer hemocytometer. Results showed that Diet-8 has significantly reduced the number of *Nosema* spores (50,000±28867.5 spores/bee) compared to the control group of colonies (5,50,000±28867.5 spores/bee). Diet-8 was more effective than other diets, suggesting that plant-based pollen substitute diets can help effectively manage Nosema infections and maintain healthy and disease-free honeybee colonies throughout the year.

Keywords: *Apis mellifera* L, Medicinal plants, Microsporidian parasite, Phytochemicals, Pollen substitute diet

INTRODUCTION

The Western honeybee *Apis mellifera* L. is a vital pollinator, contributing billions of dollars to global agricultural production annually (Kulhanek *et al.,* 2021). However, honeybee populations have declined in recent decades, largely attributed to a combination of factors, including pesticide exposure, infectious diseases and habitat loss (Kulhanek *et al.,* 2021). One of the most significant pathogens affecting honeybees is the microsporidian parasite *Nosema ceranae*, which can severely impact individual bee health and colony survival (Jousse *et al.,* 2020). *Nosema* infections disrupt gut function, reduce lifespan and impair colony-level behaviours critical for pollination services (Jousse *et al.,* 2020).

Nosema is a microsporidian, a taxonomic category of single-celled protozoan parasites that infect a variety of animal hosts. Honeybees develop the disease by swallowing *Nosema* spores via food sharing or consuming faeces from an infected nestmate. Once ingested, the *Nosema* replicates inside midgut (stomach) cells and seizes the nutrition of the honeybee. The midgut cell membrane eventually ruptures due to the *Nosema* spore's subsequent rapid multiplication. This releases more spores into the honeybee's midgut and increases the likelihood that the disease will be transmitted to other bees. The symptoms of *Nosema apis* infection are, large numbers of dead bees occurring in the colony and diarrhoea stains at entrances of the hive, indicating gastrointestinal disorders (Mortensen *et al.,* 2020; Bourgeois *et al.,* 2010; Araneda *et al.,* 2015). In temperate areas, *N. apis* infections generally peak in the spring, reduce during the summer and rise again in the fall before lowering in the early winter (Higes *et al.,* 2010). The prevalence of Nosemosis varies with location and season of year (Mulholland *et al.,* 2012). The lifespans of infected honeybees are reduced and they frequently exhibit extreme hunger, i.e., persistently requesting to be fed by other adult honeybees (Mayack and Naug, 2009). Because *Nosema* infections may not be found outside until the colony is significantly depleted, beekeepers may find the danger of infection particularly distressing.

Beekeepers commonly provide pollen supplements to bee colonies when they believe that bees suffer from a lack of nutrition or the incoming resources are of poor or inadequate quality. For example, pollen diets, which often contain a protein source derived from soy, wheat, brewers' yeast, milk powder and legumes enriched with vital vitamins, are frequently fed to migratory colonies when they are utilized to pollinate crops. These diets significantly contribute to the colony's protein requirements (DeGrandi-Hoffman *et al.,* 2010).

Medicinal plants having antimicrobial properties have been explored as potential natural treatments for *Nosema* (Alberoni *et al.,* 2023). An alternative strategy is to investigate the usage of plant-based pollen substitutes as a dietary intervention to boost honeybee immunity and resistance to *Nosema* (Daisley *et al.,* 2020).

Selected medicinal plants have essential phytochemicals and other protein-rich ingredients for inhibiting the growth of *Nosema* spores. Phytochemicals play an important role in honeybee health, and plant-derived compounds may be able to inhibit *Nosema* spore development (Daisley *et al.,* 2020). Phytochemicals such as caffeic, gallic, p-coumaric acids, quercetin, thymol, resveratrol and kaempferol most effectively prolonged the honeybee's lifespan (Bernklau *et al.,* 2019).

Thymol inhibits the growth of pathogenic bacteria and fungi (Rice, 2001; Yücel & Doğaroğlu, 2005). Resveratrol is a phytoalexin produced by certain plants in response to infections caused by phytopathogenic microorganisms (Fremont, 2000; Prokhoda *et al.,* 2019). The present study aimed to develop plant-based pollen substitute diets for controlling of *Nosema* spore infection in *Apis mellifera* L. colonies.

MATERIALS AND METHODS

The study was conducted at Dr. Babasaheb Ambedkar Marathwada University, Campus, Chhatrapati Sambhajinagar. Feeding experiments were carried out on honeybee colonies (*Apis mellifera* L). Plant-based pollen substitute diets were fed to honeybee colonies from July 01, 2023, to August 10, 2023. Temperature ranges from 20.0ºC to 30.0ºC, while humidity ranges from 73.8% to 96.3%. Sixteen honeybee colonies were selected for the experimentation. All colonies were standardized, each consisting of eight frames, and all maintained the same strength as worker bees. A frame feeder was provided for feeding treatments.

The leaves and fruits of selected plants were collected from Chhatrapati Sambhajinagar at Latitude: 19.9007° N Longitude: 75.3116° E. The fine leaf powders of plants, such as coriander (*Coriandrum sativum*), moringa (*Moringa oleifera*), tulsi (*Ocimum sanctum*), lemongrass (*Cymbopogon citratus*) and fruit powders of amla (*Embilica officinalis*) and guava (*Psidium guava*) were prepared. The selection of plants to prepare pollen substitute diets was based on phytochemicals. These fine powders of the above plant leaves and fruits were mixed with other common ingredients separately and prepared seven feed combinations, labeled as Diet -2 to Diet-8 (Table 1). White crystal sugar was used to prepare sugar syrup. Sugar and water were used in a 1:1 ratio (i.e., 50% sugar and 50% water), then boiled for 4 to 5 minutes and filtered.

Fine powders of coriander, moringa, tulsi and lemongrass leaves and fruit powders of amla and guava were added separately together with other ingredients such as defatted soya flour, skimmed milk powder, brewer's yeast, powdered sugar, turmeric powder, vitamins A, D, E & K, citric acid, guar gum powder, potassium sorbate (E202), sodium propionate (E281) as shown in Table 1.

Method of feeding

A total of sixteen colonies were selected for experimentation, each with eight frames, fully covered with bees on both sides. The total number of bees per frame was = 4000. Therefore, considering the previous assessment, the total number of bees was = 08 X 4000 = 32,000 bees/colony. These colonies were assigned to eight groups, each group with two colonies. Sixteen colonies were allotted as eight groups; each group contained two colonies. Colonies were then labelled as Diet-1 (Control), Diet-2 (Coriander), Diet-3 (Moringa), Diet-4 (Tulsi), Diet-5 (Lemongrass), Diet-6 (Amla), Diet-7 (Guava) and Diet-8 (Mixed diet). The feeders of all the colonies were washed with water and sun-dried properly. The 10 gm of diet powder + 100 ml sugar syrup (50%) this proportion of diet was provided separately for seven different groups of colonies. The first group of colonies was fed with Diet-1 (Control) 50% sugar syrup. a second group of colonies was fed Diet-2, a third group of colonies was fed Diet-3, a fourth group of colonies was fed Diet-4, a fifth group of colonies was fed Diet-5, a sixth group of colonies was fed Diet-6, a seventh group of colonies fed with Diet-7 and an eighth group of colonies fed with Diet-8. All diets were provided at 07.00 P.M. every day from July 01, 2023 to August 10, 2023.

Method of *Nosema* spore counting:

The honeybee samples for *Nosema* spore count were collected from the control and each experimental colony. From each frame, 05 bees were collected randomly, regardless of age. A total of 40 worker bees were collected from each colony (05 bees X 08 frames = 40 bees per colony) and preserved temporarily in Isopropyl alcohol in a plastic screw-cap container. Each sample container was labeled with the date of collection and colony code.

From each colony sample, 40 honeybees with 10 mL of distilled water were crushed using a mortar and pestle, and then 30 mL of distilled water (1 mL of water per bee) was added (Abdel-Baki *et al.,* 2016).

The crushed bees' samples was then transferred to a 100 ml beaker. Then a few drops of crushed samples were loaded on an improved Neubauer hemocytometer using a micropipette (every time the tip was replaced) and observed under a compound microscope at 400X magnification.

Then, out of 25 blocks of the Neubauer hemocytometer, only five blocks without debris were selected for counting *Nosema* spores and the total number of *Nosema* spores in the chamber grid was counted (Mortensen *et al.,* 2020). *Nosema* spores were counted four times at intervals of 8 days. The first count was done before feeding the artificial diet as an initial reading. Subsequent counts were performed 8, 16, 24 and 32 days after feeding.

Calculation of the *Nosema* spore count

The measurement of observed *Nosema* spore was done using a Neubauer hemocytometer and estimation of counted *Nosema* spore was done using formulae and found several spores per bee (Observed spore count from 05 blocks multiplied by 04 million divided by 80 squares counted is equal to the number of spores per bee.

Nosema spores counted from 5 blocks, each with 16 squares (5 × 16 = 80). If those 05 blocks were 39 × 4,000,000/ 80= 19,50,000 (or 1.95 million) spores per bee using the formula.

Number of spores/bees =

$$\frac{\text{Raw spore count from 5 blocks} \times 4{,}000{,}000}{\text{No. of squares counted}} \qquad \text{(Eq. 1)}$$

$$\text{No. of spores/bee} = \frac{00 \times 4{,}000{,}000}{80} = 50{,}000 \text{ spore/bee}$$

(i.e., 50.000 was considered as zero (Nil) spores per bee)

Statistical analysis

Analysis of data with one-way ANOVA using the Post Hoc-Duncan multiple range test (DMRT) by SPSS 29.0.2.0. The results were denoted as (Mean ± Standard Error) and the separation of means described with

Table 1. Composition of plant-based pollen substitute diets (/100 gm)

Diet Codes	DSF	SMP	BY	PS	TP	Vit.	CA	GGP	E202	E281	CLP	MLP	TLP	LLP	AFP	GFP
Diet-1 Control	1 Liter of 50 % Sugar syrup															
Diet-2	45g	15g	6.4g	20g	0.7g	0.8g	5.0g	0.5g	0.3g	0.3g	6.0g	-	-	-	-	-
Diet-3	45g	15g	6.4g	20g	0.7g	0.8g	5.0g	0.5g	0.3g	0.3g	-	6.0g	-	-	-	-
Diet-4	45g	15g	6.4g	20g	0.7g	0.8g	5.0g	0.5g	0.3g	0.3g	-	-	6.0g	-	-	-
Diet-5	45g	15g	6.4g	20g	0.7g	0.8g	5.0g	0.5g	0.3g	0.3g	-	-	-	6.0g	-	-
Diet-6	45g	15g	6.4g	20g	0.7g	0.8g	5.0g	0.5g	0.3g	0.3g	-	-	-	-	6.0g	-
Diet-7	45g	15g	6.4g	20g	0.7g	0.8g	5.0g	0.5g	0.3g	0.3g	-	-	-	-	-	6.0g
Diet-8	45g	15g	6.4g	20g	0.7g	0.8g	5.0g	0.5g	0.3g	0.3g	1.0g	1.0g	1.0g	1.0g	1.0g	1.0g

Abbreviations: DSF: Defatted Soya flour, SMP: Skimmed milk powder, BY: Brewer's yeast, PS: Powdered sugar, TP: Turmeric powder, Vit.: Vitamin A, D, E & K, CA: Citric acid, GGP: Guar gum powder, E202: Potassium sorbate, E281: Sodium propionate, CLP: Coriander Leaves Powder, MLP: Moringa Leaves Powder, TLP: Tulsi Leaves Powder, LLP: Lemongrass Leaves Powder, AFP: Amla fruit powder, GFP: Guava fruit powder homogeneous subsets of means was noted by small alphabetical letters. The same letters meant 'No significant difference' among the means of subsets. P < 0.05 indicates the significant differences among the means in those subsets.

RESULTS AND DISCUSSION

The effect of plant-based pollen diet treatments on the rate of *Nosema* infection was observed. The infection rate was calculated before feed treatment and after feeding at 8-day intervals up to 32 days until the spores disappeared totally from the bees as shown in Table 2 and Fig. 1. The highest *Nosema* spore count was recorded in control colonies fed with Diet-1 (5,50,000±28867.5 spore/bee) after 32 days of feeding. The lowest rate of *Nosema* spore count was seen in the experimental group of colonies fed with Diet-8 (50,000±28867.5 spore/bee) as compared to control Diet-1 and other diets. However, Nosema spores were significantly reduced (all *p* values < 0.05). It was observed that feeding only sugar syrup was not adequate and healthy for the bees. A lowered rate of *Nosema* spore count was observed in colonies fed with Diet-8 (4,00,000±28867.5 spore/bee) after 24 days and *Nosema* spores were found to disappear totally after 32 days of feeding Diet-8 (50,000±28867.5 spore/bee), with a significant decrease in *Nosema* spores (all *p* values < 0.05). This value (50,000 *Nosema* spores/bee) is considered zero spores count in honeybees. The performance of Diet-4, containing tulsi leaf powder (2,50,000±28867.5 spore/bee) was found to be best in decreasing the infection rate significantly compared to the other diets except Diet-8. The Diet-2 containing coriander leaf powder (3,00,000±28867.5 spore/bee) and Diet-3 with moringa leaf powder (3,00,000±28867.5 spore/bee) have shown similar *Nosema* spore counts with significantly lowered rate of *Nosema* infection after 32-days of feeding with a significant decrease in *Nosema* spores (all *p* values < 0.05). Similarly, Diet-5 containing lemongrass leaf powder (3,50,000±28867.5 spore/bee) and Diet-6 containing amla fruit powder (3,50,000±28867.5 spore/bee) have shown similar *Nosema* spore counts with a significant decrease of *Nosema* spores (all *p* values < 0.05). The Diet-7 containing guava fruit powder (4,00,000±28867.5 spore/bee) has shown the lowest response in decreasing *Nosema* spore count as compared to Diet-2, Diet-3, Diet-4, Diet-5 and Diet-6 after 32 days of feeding with a significant decrease in *Nosema* spores count (all *p* values < 0.05).

The genus *Nosema* produces microspore-forming parasites that cause Nosemosis, a disease that affects worker bees, queens and drones by attacking the middle intestine's epithelial lining (Botías *et al.*, 2012; Bollan *et al.*, 2013). The unicellular microsporidium *Nosema apis* was the only recognized causative agent of Nosemosis in honeybees (*Apis mellifera*) (Nabian *et al.,* 2011). The *Nosema ceranae* can infect honeybee larvae and reduce longevity in adult bees (Eiri *et al.,* 2015; Palmer-Young et al., 2017). According to Porrini *et al.,* 2011, Rinderer and Dell Elliott, 1977 certain proteins can raise Nosemosis. However, in the commercial diets they tested, there was no correlation between the protein content and the observed *Nosema* levels in the bees.

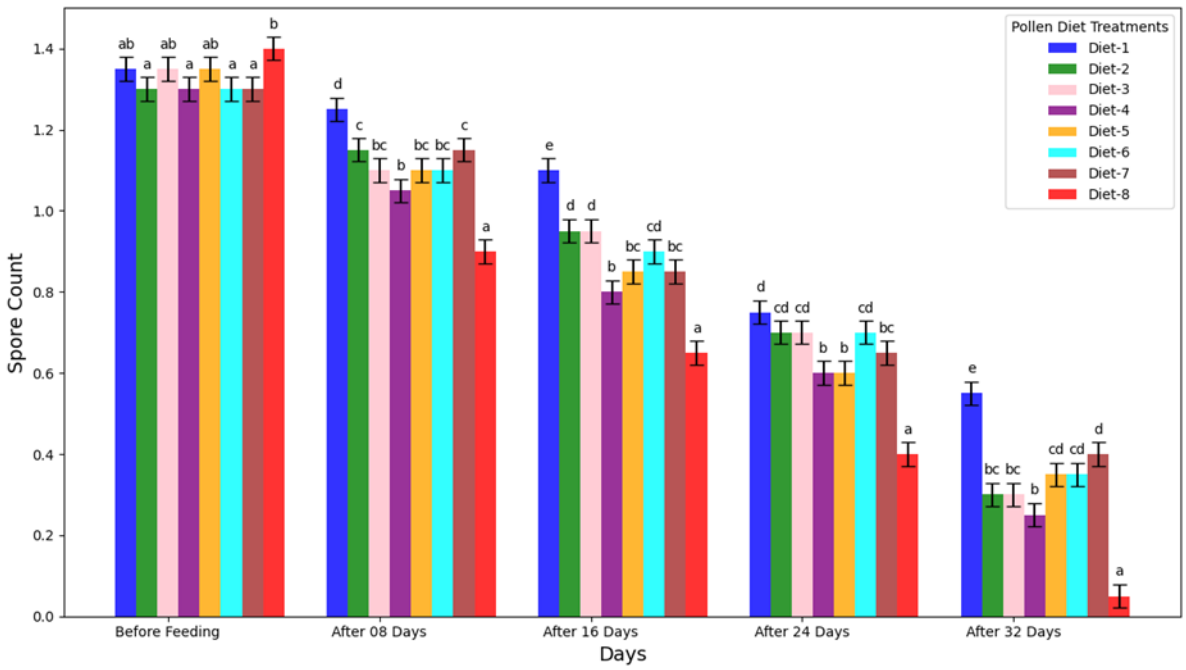

Fig. 1. *Showing the effect of pollen diet treatments on rate of infection of Nosema spores*

Table 2. Showing the effect of pollen diet treatments on the *Nosema* spore count in honeybees. (Number of spores/ bee)

Days	Diet-1 (Control)	Diet-2	Diet-3	Diet-4	Diet-5	Diet-6	Diet-7	Diet-8
No. of spores before feeding	1,350,000 ± 28867.5[ab]	1,300,000 ± 28867.5[a]	1,350,000 ± 28867.5[ab]	1,300,000 ± 28867.5[a]	1,350,000 ± 28867.5[ab]	1,300,000 ± 28867.5[a]	1,300,000 ± 28867.5[a]	1,400,000 ± 28867.5[b]
After 08-days	1,250,000 ± 28867.5[d]	1,150,000 ± 28867.5[c]	1,100,000 ± 28867.5[bc]	1,050,000 ± 28867.5[b]	1,100,000 ± 28867.5[bc]	1,100,000 ± 28867.5[bc]	1,150,000 ± 28867.5[c]	9,00,000 ± 28867.5[a]
After 16-days	1,100,000 ± 28867.5[e]	9,50,000 ± 28867.5[d]	9,50,000 ± 28867.5[d]	8,00,000 ± 28867.5[b]	8,50,000 ± 28867.5[bc]	9,00,000 ± 28867.5[cd]	8,50,000 ± 28867.5[bc]	6,50,000 ± 28867.5[a]
After 24-days	7,50,000 ± 28867.5[d]	7,00,000 ± 28867.5[cd]	7,00,000 ± 28867.5[cd]	6,00,000 ± 28867.5[b]	6,00,000 ± 28867.5[b]	7,00,000 ± 28867.5[cd]	6,50,000 ± 28867.5[bc]	4,00,000 ± 28867.5[a]
After 32-days	5,50,000 ± 28867.5[e]	3,00,000 ± 28867.5[bc]	3,00,000 ± 28867.5[bc]	2,50,000 ± 28867.5[b]	3,50,000 ± 28867.5[cd]	3,50,000 ± 28867.5[cd]	4,00,000 ± 28867.5[d]	50,000 ± 28867.5[a]

Data on the same alphabet means, 'No significant difference' among the means of subsets; P < 0.05 indicates the significant differences among the means in those subsets

In the present study, a plant-based pollen diet was rich in essential phytochemicals that significantly improved overall health, improved immunity and lowered micro-spore count. The results showed that plant-based pollen substitute diets have significantly decreased spores after feeding (all *p* values < 0.05). The medicinal plants have phytochemicals (i.e., phenol, flavonoid, alkaloid, terpenoid, p-coumaric acid and quercetin) that are responsible for the decreased rate of *Nosema* infection. The results of the present study correlated with (Maistrello *et al.,* 2008), who also used medicinal plants with phytochemicals such as resveratrol (*Polygonurn cuspidatum*), lysozyme, vetiver oil (*Vetiveria zizanioides*) and thymol (*Thyme*). Phytochemicals showed positive effects, but they cannot show any toxic impact on adult bees and have a high consumption rate. All the compounds show significant effects in treating *Nosema* disease. Therefore, plant-based pollen diets were found to be a suitable treatment against *Nosema* disease.

Medicinal plants such as coriander, moringa, tulsi, lemongrass, amla and guava have almost all types of phytochemicals (phytosterol, phenolic acid, flavonoid, alkaloids, terpenoids, p-coumaric acids, caffeic acid, gallic acid and quercetin) that fulfill the requirement of nutrition and disease resistance capacity (Bhat *et al*., 2014; Gopalakrishnan *et al*., 2016; Singh and Chaudhuri, 2018; Vidhani *et al*., 2016; Asaolu *et al*., 2009; Mishra and Mahanta, 2014; Sachan *et al*., 2013; Tanwar *et al*., 2014). Phytochemicals such as caffeic acid, gallic acid, p-coumaric acids and kaempferol prolonged the honeybee's lifespan most effectively. It is necessary to boost growth, improve immunity and increase the resistance of bees against diseases (Bernklau *et al*., 2019).

The decreased *Nosema* spore counts correlate significantly (all *p* values < 0.05) with the overall performance and results of Bhat *et al*. (2014); Gopalakrishnan *et al*. (2016); Singh and Chaudhuri (2018); Vidhani *et al*. (2016); Asaolu *et al*. (2009); Mishra and Mahanta (2014); Sachan *et al*. (2013); Tanwar *et al*. (2014); Bernklau *et al*. (2019).

Analysis of variance (ANOVA) showed a significant difference ($p < 0.05$) in *Nosema* spore count among the different *Apis mellifera* L. colonies fed with plant-based pollen substitute diets and the control colonies. The various small alphabet letters within every column of Table 2 represent *Nosema* spore counts with statistically significant differences (P < 0.05).

Conclusion

This study has evaluated the impact of plant-based pollen substitute diets on the *Nosema* spore infection rate in *Apis mellifera* L. colonies. The number of spores per Diet treatment can vary based on bee nutrition. Plant-based pollen diets containing proteins and essential phytochemicals influenced microspores and reduced the *Nosema* infection. Diet-8, containing equal quantities of all the medicinal plants, lowered the number of *Nosema* spores observed in *A. mellifera* L. The mean differences are denoted as small alphabetical letters within every column representing statistically significant differences (P < 0.05). In addition, all ingredients such as defatted soya flour, skimmed milk powder, brewer's yeast, powdered sugar; turmeric powder; vitamins A, D, E and K; citric acid, guar gum powder; potassium sorbate and sodium propionate; the leaf and fruit powders of selected medicinal plants including leaf powders of coriander, moringa, tulsi, lemongrass and fruit powders of amla and guava with 50% sugar syrup have shown significant results in decreasing *Nosema* spore count and also to enhance immunity and overall efficiency of bees as compared to the control diet. Nutritionally, medicinal plants may fulfil the requirement of all phytochemicals essential for growth, reproduction and maintaining resistance against diseases. Hence, mixed Diet-8 can be recommended for the commercial beekeeping practice to improve honeybee colonies' health and overall performance.

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Conflict of interest

The authors declare that they have no conflict of interest.

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