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Zeolite clinoptilolite as a dietary supplement and remedy for honeybee (*Apis mellifera* L.) colonies

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ABSTRACT: Control of the nosema disease poses a major challenge, and therefore, treatment of this serious parasitic disease using natural preparations could be of great benefit. The aim of this study was to test the performance of zeolite clinoptilolite as a curative measure against honeybee colonies (*Apis mellifera* L.) naturally infected by *Nosema ceranae*. The histopathological structure, and the content and distribution of mucosubstances and histochemical activity of aminopeptidase and non-specific esterase in the midgut mucosa of honeybees originating from colonies fed sugar syrup supplemented with zeolite minerals was studied. A decline in the number of spores in honeybees fed with zeolite clinoliptolite was observed on the first sampling day (Day 10; 53.25 ± 15.15 million spores/bee), though a statistically lower number of spores in comparison to the control was confirmed on Day 20 (41.08 ± 9.4 million spores/bee), Day 30 (28.42 ± 7.79 million spores/bee) and Day 40 (24 ± 6.25 million spores/bee). The possibility of using natural zeolites as a dietary supplement for honeybee colonies as a preventative measure and for the reduction of the deleterious effects of nosemosis is discussed.

Keywords: *Apis mellifera*; *Nosema ceranae;* zeolite clinoptilolite; dietary supplement

Honeybees contribute significantly to the pollination of agricultural crops and native plants and consequently to sustainable agriculture dependent on biodiversity. This, alongside honey production, makes these insects both ecologically and economically important. Several factors can affect the vitality of honeybee colonies, including disease (Murilhas 2002; Higes et al. 2010), availability of adequate food resources (Naug and Gibbs 2009), weather conditions and the size of the adult bee population and brood quantity and quality. Nosemosis type C is caused by the microsporidium *Nosema ceranae* (Higes et al. 2006), a dominant and highly prevalent honeybee disease worldwide. It has negative impacts on honey production and beekeeping profitability, and is a serious threat to apiculture through the possible sudden collapses of colonies or shortened lifespan due to the energetic stress of individual adult bees (Higes et al. 2009; Mayack and Naug 2009; Botias et al. 2013). Currently, due to the asymptomatic nature of the disease, its control is difficult (Martin-Hernandez et al. 2007). Fumagillin is an antimicrobial agent isolated in 1949 from *Aspergillus fumigatus* (Hanson and Eble 1949), and has been the only widely used treatment for nosemosis for few decades (Higes et al. 2011). As has been recently reported however, fumagillin may exacerbate, rather than suppress, *N. ceranae* infection (Huang et al. 2013).

European Union regulations prohibit the use of antibiotics in the treatment of apian diseases due to the potential development of resistance, as well as the counterproductive effects of disease dissembly, possible relapses, and the presence of harmful

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antibiotic residues and their secondary metabolites in honey. The effects of residues and their by-products in honey and wax represent an environmental concern and are another reason for reducing the use of conventional chemical control methods in beekeeping. For that reason, the need arises for natural preparations and/or alternative therapies.

The natural mineral supplement Eko ZeoPet contains the volcanic mineral clinoptilolite from the zeolite family of minerals and is produced by a special procedure called micronisation, which significantly increases its bioactivity and bioavailability. As a mineral supplement to human and animal food, clinoptilolote, acts as a powerful antioxidant (Zarkovic et al. 2003; Andronikashvili et al. 2009; Dogliotti et al. 2012), enhances the general condition and immunity of an organism (Pavelic et al. 2002; Ivkovic et al. 2004), improves the bio-efficiency of food and essential minerals (Tsitsishvili et al. 1999; Yannakopoulos et al. 2000), promotes healing of skin damage (Bedrica et al. 2003) and can have anti-cancer and antimicrobial effects (Pavelic et al. 2001; Grce and Pavelic 2005; Muck-Seler and Pivac 2003).

Proteolytic enzymes secreted by the midgut epithelial cells of honey bees (Malone and Gatehouse 1998) are extremely important for the digestion of pollen, which is the main source of protein in the honeybee diet (Brodschneider and Crailsheim 2010). Leucine-aminopeptidase (LAP) belongs to the group of proteolytic enzymes, the activity of which leads to the degradation of proteins into amino acids. Esterases are hydrolysing enzymes that degrade esters into acids and alcohol during the chemical reaction of hydrolysis. Two groups of substrates for degradation are distinguished: one in which sugars represent an "acid", such as pectin-methyl ester, and a second in which sugars behave as alcohols, such as xylan (Bitondi and Mesteriner 1983; Mackert et al. 2008). The activity of these enzymes is the strongest in young bees, and these enzymes are necessary for the degradation of proteins and esters, which are essential for building an entire honeybee organism and for physiological function, especially through the development of glandular tissue (Winston 1987).

We hypothesised that the Eko ZeoPet mineral supplement, if added to the honeybee diet, could improve the healing of micro-damage in the midgut epithelium of bees infected with *N. ceranae* spores, reduce the number of spores and improve the immune status of the honeybee colonies. The aim of this study was to determine the impact of supplemental feeding of honeybee colonies with the mineral supplement Eko ZeoPet on the number of *Nosema* spp. spores, on the histopathological structure, and the content and distribution of mucosubstances and histochemical activity of aminopeptidase and non-specific esterase in the mid-gut mucosa of honeybees originating from the treated colonies.

MATERIAL AND METHODS

Honeybee colonies and assessment. The field experiment was conducted over a 40-day period (beginning 4 August 2013) at an apiary situated in continental Croatia (45°56'44''N, 16°36'20''E), after the harvesting season. In order to perform the field assay, 24 homologous honeybee colonies, naturally infected with *N. ceranae* and accommodated in standard LR hives acquired from the same beekeeper, were selected and divided into experimental (12) and control (12) groups. At the beginning of the study, none of the colonies showed clinical signs of brood diseases and the last treatment of *Varroa destructor* invasion was carried out on 25 July 2012 (CheckMite®, a.m. cumaphos) to avoid the negative effects of parasitisation on colony health. During clinical inspection of honeybee colonies, approx. sixty forager bees per colony were collected from the hive entrance for microscopic examination for the presence of *Nosema* spp. spores, and for multiplex PCR analyses (Tlak Gajger et al. 2010) for species determination. Clinical signs of disease, the presence of the queen and honeybee mortality were checked on every visit to the experimental apiary. No insecticides were in use in the area surrounding the apiary (radius approx. 3 km) during the experiment.

Field assay procedure and feeding. The experimental group of honeybee colonies were fed with 250 ml sugar syrup (1 : 1 water-sugar; Viro sugar, Croatia) with the addition of 5 g zeolite clinoptilolite mineral (Eko Zeo Pet – food supplement for animals, Velebit I. International d.o.o., Croatia). The dose was modified according to manufacturer instructions for use of this mineral supplement in the diet of pets with a body weight of 10 to 20 kg. The control group of honeybee colonies received only sugar syrup prepared in the same manner. The treated sugar syrup, and the pure sugar syrup, was

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administered to the honeybee colony in feeders situated under the roof of the hives, over 20 consecutive days.

Determination of infection level and Nosema species. Each sample consisted of approximately 60 adult honeybees (foragers) taken at the hive entrance on Days 10, 20, 30 and 40 after the initial sampling (conducted prior to the first feeding session). Bee samples were collected into clean plastic receptacles at noon. Honeybees were counted in each cumulative sample, their abdomens were separated and thoroughly crushed and homogenised in a plastic container containing 1 ml water per bee. *Nosema* spp. spores were counted in each sample using a Burker-Turk haemocytometer, and the infection level was calculated according to Cantwell (1970). Each numbering procedure was replicated three times. The counting equipment was carefully washed after each sample counting in order to avoid contamination with spores from the previous sample. Extraction of genomic DNA and further molecular analysis was performed as described elsewhere (Tlak Gajger et al. 2010).

Histochemical analyses. In total, twenty bees were collected from each testing colony on Day 30 after initial feeding, and the intestines of each honeybee were extracted after brief exposure to low temperature (10 min at 4 °C). For extraction purposes, a larger pair of forceps was used to hold the head and the thorax of each honeybee, and a smaller pair of forceps to hold the top of the last abdominal segment and carefully pull out the intestines. Immediately after, the front (honey sac) and rear (rectum) part of the gut was cut off. Midguts were fixed in a 4% formaldehyde solution, dehydrated in 96% ethanol, inserted into paraffin blocks and sliced into 6 to 7 µm thick sections with a microtome. Dewaxed sections were stained for general morphological purposes according to the Hemalaon-Eozinic method (HE; Roulet 1948). For the purposes of describing neutral (hexose-containing) mucosubstances, acid and sulphate mucosubstances and metachromasia, the Periodic Acid-Schiff Reaction (PAS; McManus 1948), Alcian blue (pH = 2.5; Mowry 1956), Alcian blue (pH = 1.0; Lev and Spicer 1964) and Toluidine blue (TB)-specific staining (Pearse 1968) was used.

For examination of enzyme activity, midgut samples were fixed in glass tubes with a chilled (4 °C) solution of formol-calcium for 24 h in the refrigerator. This solution was then taken out and cooled sucrose was added. Prepared samples were stored at 4 °C until further processing. Degreased cuts of midgut were prepared and dyed with special stains for determination of leucine aminopeptidase (LAP) (Hrapchak and Shennan 1980) and esterase activity (Burstone 1962). The level of enzyme activity was determined using qualitative microscopic examination and histological preparations were analysed as described by Tlak Gajger et al. (2013).

Microscopic examination was performed under a bright field microscope Olympus Bx41, at 400 × magnification for natural preparations, and 10 to 40 × magnification for histological preparations. The photographic records of the preparations were taken using an Olympus DP12 U-TVO camera.

Statistical analysis. In order to assess and verify the differences in the spore load data between the experimental and control group on different sampling dates, one-way analysis of variance (ANOVA) and Mann-Whitney *U* test were performed using the statistical software package Statistica-StatSoft v.7. The number of spores in honeybee samples collected on four sampling dates was compared per group (experimental, control) and sampling date (Days 10, 20, 30, 40) using one-way ANOVA, while the two-sample comparisons were carried out using the nonparametric Mann-Whitney *U* test in order to compare the mean values between groups. Statistical significance testing was conducted using a significance level of $\alpha = 0.05$ to define statistical differences (0.95 confidence interval).

RESULTS

Molecular analyses performed using multiplex PCR on all honeybee samples showed infection with *N. ceranae* spores. Control and experimental honeybee colonies consumed the complete amount of offered sugar syrup. The results of spore loads measured on the sampled honeybees are shown in Figures 1a and 1b, and Table 1.

A continuous decline in the *Nosema* infestation in bees fed with sugar syrup supplemented with Eko ZeoPet on given sampling dates (days) was confirmed by the Mann-Whitney *U* test, as presented in Table 1. A decline in the number of spores in honeybees fed with zeolite clinoliptolites was already observed on the first sampling date (Day 10) ($U = 19.5$; $P = 0.0024$), though a statistically lower number of spores in comparison to the control was

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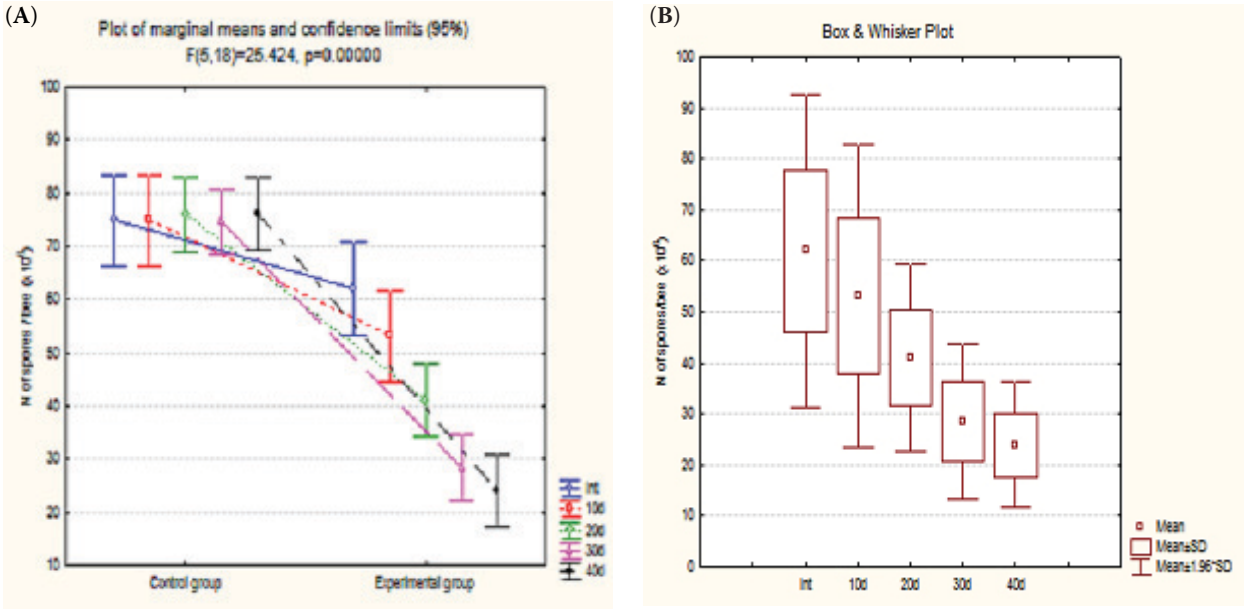

Figure 1. Differences in the number of spores between experimental and control group by sampling date (**1A**); decreasing trend of the number of spores by sampling dates within the experimental group (**1B**)

confirmed for each subsequent sampling date, on Day 20 (U = 0; P = 0.000041), Day 30 (U = 0; P = 0.000032) and Day 40 (U = 0; P = 0.000032), respectively. Also, in the experimental group reduced numbers of spores compared to the initial spore count at an average of 16.94% on Day 10; 26.22% on Day 20; 51.21% on Day 30 and 58.82% on Day 40 after the first feeding, were determined.

The histological examination of the midgut preparations of honeybees in which *Nosema* spp. spores were not coprologically found, revealed the presence of all layers of the ventricular wall, i.e. outer longitudinal and transverse muscle layer, and median basement membrane, coated with a layer of highly cylindrical epithelial cells among which individual regeneratory cells were visible, and rabdorium and a partly peeled perithrophic membrane next to the ventricular lumen. In the histological preparations of the midgut of bees infected with *N. ceranae* spores, degenerative changes and lytic processes were found within the epithelial cells. Depending on the severity of the invasion and the consequent high osmotic pressure due to the presence of a large number of spores, destroyed epithelial cells were also found.

Histochemical analysis showed that the midgut mucosa content included glycogen and/or oxidable dioles (PAS+). In diseased honeybees fed with sugar syrup supplemented with Eko ZeoPet, the amounts of neutral mucosubstances were elevated (Figure 2) in comparison with diseased non-treated bee midguts. Zeolite treatment of diseased honeybees led to visible and significant increases in AB (pH = 2.5) positive mucosubstances (Figure 3), or mucosubstances with carboxyl groups, sialic acid or uranic acid and/or with sulphate esters. Meanwhile, the O-sulphated esters of mucosubstances (AB, PH = 1.0) in the mucous coat of the intestine did not show significant changes in structure in comparison to non-treated honeybees. Metachromatic-stained (TB) non-sulphated mucosubstances containing sialic acid were visible

Table 1. Mean number of Nosema spores per bee ($\times 10^6$) ± standard deviation (SD) collected on four sampling dates/days; different letters after values at the end of each denotation indicate that the values are significantly different at $P < 0.05$ level after Mann-Whitney U test

Group	Day				
	1	10	20	30	40
Control group	74.83 ± 13.15^a	74.92 ± 13.27^a	76 ± 13.46^a	74.58 ± 12.54^a	76 ± 15.14^a
Experimental group	62.00 ± 15.69^a	53.25 ± 15.15^a	41.08 ± 9.4^b	28.42 ± 7.79^b	24 ± 6.25^b

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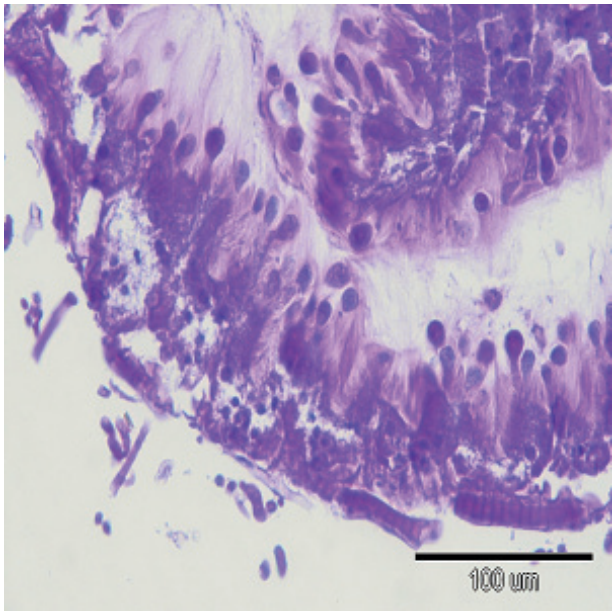

Figure 2. Midgut, honeybee infected with *N. ceranae* spores fed with sugar syrup supplemented with Eko ZeoPet; PAS, magnification × 40.

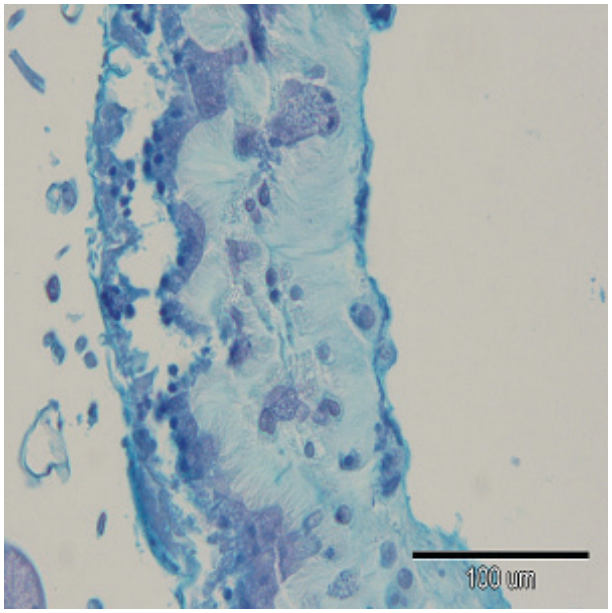

Figure 3. Midgut, bee infected with *N. ceranae* spores fed with sugar syrup supplemented with Eko ZeoPet; AB 2.5, magnification × 40

in the apical part of epithelial cells and in the apical mucosa coat. In the midguts of diseased honeybees originating from the experimental group fed with Eko ZeoPet, increasing amounts of non-sulphated mucosubstances were clearly visible in the upper mucosa stratum.

In all midgut samples of honeybees originating from the experimental group, strong and visible LAP activity was found, which was particularly noticeable in the apical parts of the epithelial cells. In the midguts of bees sampled from the control group, LAP activity was weak. The described

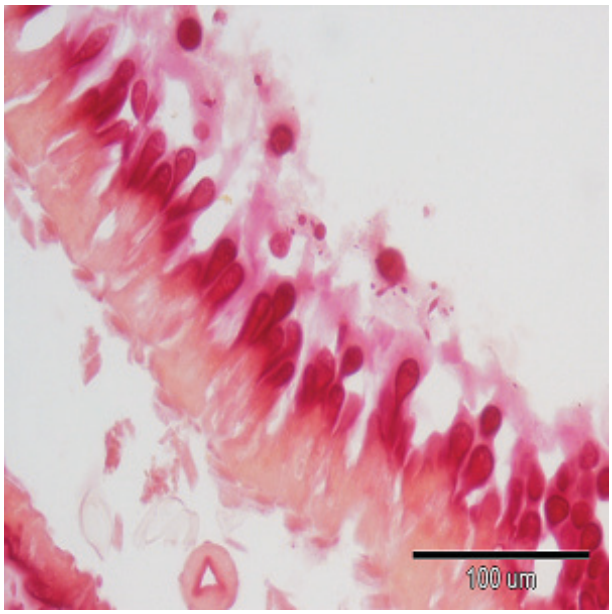

Figure 4. Strong LAP enzymatic activity in enterocytes in the midgut of honeybee colonies previously fed sugar syrup supplemented with Eko ZeoPet; magnification × 40

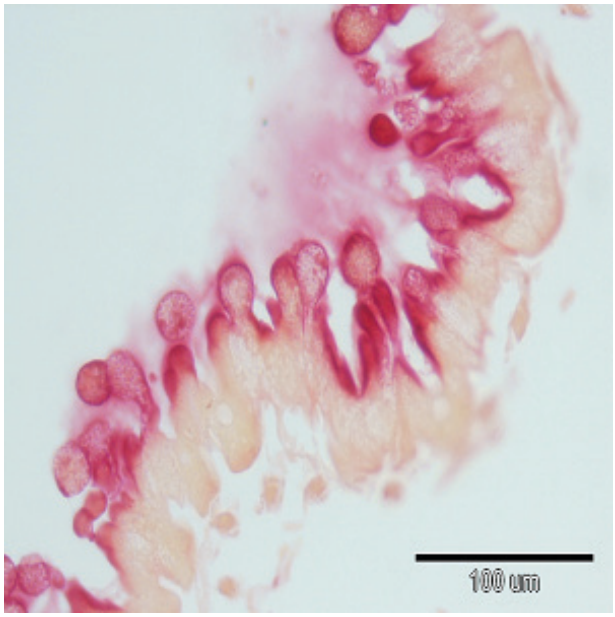

Figure 5. LAP activity in the midgut of untreated honeybee colonies; magnification × 40

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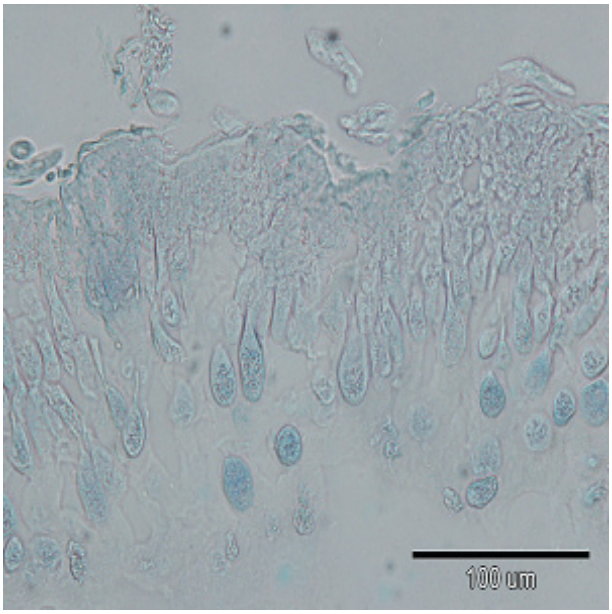

Figure 6. Weak esterase enzymatic activity in the midgut of honeybees originating from colonies previously fed sugar syrup supplemented with Eko ZeoPet; magnification × 40

results are shown in Figures 4 and 5. We found moderate-to-weak esterase activity in the midgut preparations of honeybees from the experimental group, which was more visible in the area of the rabdorium and partly in the lumen of the ventriculum itself (Figure 6). Esterase activity in the midgut preparations of honeybees from the control group was negative to barely visible.

DISCUSSION

Natural zeolites hold a special place in modern medicine due to attributes that combine alternative and *state of the art* medicine (Jurkic et al. 2013). They can exert many therapeutic properties effects to their activation by the advanced technology of tribomehanic micronisation, which enables an increase in the active surface area, thus providing greater bioavailability (Kralj and Pavelic 2003). There have been no reports of any toxic effects of zeolite clinoliptolites on animals or humans. Because *N. ceranae* may have a deleterious effect at the colony level (Higes et al. 2008) and some non-treated colonies die leaving no adult honey bees in the hive (Botias et al. 2013), there is a real need for the application of alternative treatments and beekeeping techniques to combat this emerging disease.

The results of this study demonstrated that zeolite clinoptilolite in the form of the Eko Zeo Pet supplement administrated via sugar syrup clearly reduced the development of the microsporidium *N. ceranae* in the honeybee midgut. This was observed in the lower spore counts compared to control groups observed from the initial time point on Day 10 and also on Days 20, 30 and 40 of the field experiment, when spore loads were significantly decreased ($P < 0.05$). Given that analysing the percentage of forager bees infected by *Nosema* spp. spores was previously demonstrated to be a more accurate method of estimating the level of infection at the colony level (Oliver 2011; Goblirsch et al. 2013), we applied this method to examine the therapeutic capacity of Eko ZeoPet as a dietary supplement. Despite the failure to achieve a complete cure, it should be emphasised that the honeybee colonies fed with sugar syrup supplemented with clinoliptolite had a reduced number of spores compared to the initial spore count (average: 16.94% on Day 10; 26.22% on Day 20; 51.21% on Day 30 and 58.82% on Day 40 after the first feeding). In the control group, the number the spores did not change significantly through the experimental period. According to the results presented in this study, and the excellent results in experiments on biological and therapeutic effects for different disorders in animals and humans (Rodriguez-Fuentes et al. 1997; Pavelic et al. 2001; Pavelic and Hadzija 2003; Gutzmirtl et al. 2006; Katic et al. 2006), it should be acknowledged that natural zeolites hold a substantial potential for nosemosis C treatment. Several toxicological studies have proven that certain natural zeolites, e.g. clinoptiolites, are non-toxic and completely safe for use in human and veterinary medicine (Pavelic et al. 2001).

The experimental design was based on a low amount of sugar syrup as the zeolite carrier, to ensure complete consumption by the honeybee colonies. However, the optimal manner of application of dietary supplements for regular use should be adapted to the actual working conditions of the individual beekeeper (Botias et al. 2013), and other zootechnical or environmental factors.

Several other alternative products have been evaluated as possible treatments to control nosemosis in field conditions, e.g. the herbal preparation Nozevit (Tlak Gajger et al. 2009; Tlak Gajger et al. 2011), ApiHerb (Nanetti 2009), Protofil (Chioveanu et al. 2004), Nonosz (Bekesi et al. 2009)

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or animal lysozymes and essential oils extracted from *Vetiveria zizanioides* (Maistrello et al. 2008), with varying effects. Several treatments have undergone laboratory testing on experimentally infected bees, e.g. reservatrol and tymol (Costa et al. 2010), surfactin (Porrini et al. 2011), and double strained RNA homologous to the genes that encode the ADP/ATP complex (Paldi et al. 2010).

The histochemical analysis performed in this study revealed an increase in the production of neutral mucosubstances (PAS) and mucosubstances with carboxylic groups and rich in sialic acid (AB, PH = 2.5), while the increased amount of TB methachromatic substances suggests the presence of sialic acid-rich non-sulphated mucosubstances (TB) in the superficial layer of the intestinal lumen in diseased honeybees after feeding with sugar syrup supplemented with Eko ZeoPet. The function of the secreted mucous layer is very important for lubricating undigested food, and also plays a significant role in osmoregulation and transfer of proteins or amino acids, fluids and ions (Domeneghini et al. 1998; Vegetti et al. 1999). Therefore, the mucous layer protects the ventricular epithelium from mechanical injury, pathogenic infections by microorganisms and parasitic invasions. As reported by Tibbetts (1997), mucous cells containing sialoproteins and sulphoglycoproteins also promote an increase in the viscosity of secretion, which can serve as a protective barrier. Mucosubstances secreted by the honeybee midgut epithelium have also been implicated in the absorption of easily digested molecules (Grau et al. 1992) and play a very important role in the intestinal absorption process, especially after starvation during dry weather periods when the number of intestinal goblet cells increases (Kakamand et al. 2008).

The activity of the digestive enzymes of the honeybee is closely associated with the digestion processes of food and the absorption of digested nutrients, but is also dependent on age and feeding habits (Brodschneider and Crailsheim 2010). Whereas the basis of a honeybee's diet is honey, pollen and water, LAP and esterase enzymes secreted by the midgut epithelial cells of honeybees have a particularly critical role among other digestive enzymes important for intermediate metabolism (Malone and Gatehouse 1998). Malone and Gatehouse (1998) quantitatively assessed the level of the LAP enzyme using the method of Christeller and Shaw (1989). In the present study, it was assessed qualitatively, i.e. according to the intensity of colour in stained histological preparations. Varying LAP activities have been reported in healthy and diseased honeybees on different days following artificial infection (Malone and Gatehouse 1998), though data are lacking on the extent of enzymatic activity in honeybees naturally infected with *Nosema* spp. spores and those treated with natural preparations as possible alternative treatments. Recently, we reported increased LAP activity in the midgut of honeybees treated with the herbal preparation Nozevit (Tlak Gajger et al. 2013). The present study also found increased LAP activity in the midgut of honeybees fed with the Eko ZeoPet dietary supplement, which may be the result of its protective role on the midgut epithelium. According to Liu (1984), protease activity is generally weaker in the midgut of diseased honeybees, as one of the ways in which this microsporidium disrupts digestive ability is via a reduction in proteolytic capability. Also, the lysis of infected epithelial cells provides an explanation for the decrease in enzyme activity (Malone and Gatehouse 1998). Esterase activity in the midguts derived from the experimental group was determined to be moderate-to-weak, and very weak in the control group. That is not surprising as the study was conducted on adult foragers. These results suggest that the use of zeolites stimulates the activity of these proteolytic enzymes.

Accumulating evidence from preliminary studies (Toplek 2013; Tlak Gajger et al. 2014) and the first field trial on diseased honeybee colonies suggests that oral therapy with natural zeolite clinoliptolite supplementation is associated with significant therapeutic effects on nosemosis type C, without altering normal bee physiology. However, as the number of colonies per experimental group was limited, further research is necessary to clarify the proposed mechanisms of action of the zeolite clinoliptolite compounds and to confirm the promising results observed in this study.

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