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Research

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Commercial probiotic formulas Bactocell and Levucell promote spring brood production in *Apis mellifera* L. (Hymenoptera: Apidae) colonies

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Honey bees are essential pollinators for several economically important crops. In temperate countries, honey bee colonies face multiple threats during the overwintering period, such as food availability, diseases, and confinement. Beekeepers commonly use chemicals to improve colony health during winter, but these products can have a negative impact on bee health and pathogens can develop resistance to them. Thus, there is a need for further development of alternative treatments. The aim of this study was to evaluate the impact of one endogenic bacterium (*Bombella apis*) and 2 commercial probiotic formulas (Bactocell and Levucell) on colony survival, spring development, and *Vairimorpha* (formerly *Nosema)* spp. spore count. Probiotic treatments were given in 1: 1 sugar syrup in October 2017 and April 2018, once a week for 2 wk. One experimental group was given Fumagilin-B, the only product approved in Canada to prevent nosemosis, once in October. The administration of 2 commercial probiotics, Bactocell (*Pediococcus acidilactici*) and Levucell (*Saccharomyces cerevisiae boulardii*), led to a significant increase in the number of sealed brood cells in spring. None of the probiotic treatments impacted the honey bee gut load of *Vairimorpha spp.* spores. The results suggest that beneficial microorganisms can improve spring development and performance of honey bee colonies.

Key words: Vairimorpha, beekeeping, yeast, *Lactobacillaceae*, overwintering

Introduction

The honey bee (*Apis mellifera*, Linnaeus, 1758) provides essential pollination services all around the globe. Unfortunately, the beekeeping industry is threatened by increasing overwintering mortality rates across Canada (CAPA 2022) and other countries (Neumann and Carreck 2010, Potts et al. 2010, Bruckner et al. 2023). During the winter of 2021–2022, beekeepers from Québec, Canada reported a total colony loss of 48.4%, mainly caused by ineffective varroa treatment and weather, which is the highest rate observed since 2007 (CAPA 2022).

Overwintering in Canada corresponds to a period of sharp climate shift that results in the confinement of bees in the hive and lack of defecation, which significantly affects colony health (Döke et al. 2015). During this stressful period, the prevalence of the parasitic microsporidia *Vairimorpha* sp., formerly *Nosema* sp. (Tokarev et al. 2020), the pathogen causing nosemosis, increases in colonies (Fries 2009), which reduces worker lifespan and, ultimately triggers colony collapse (Higes et al. 2008). Importantly, this stressful period is associated with a reduction of the gut microbiota diversity and the increase in abundance of several bacterial strains associated with dysbiosis (Bleau et al. 2020). Increased abundance of noncore bacterial strains belonging to the Enterobacteriaceae family is positively correlated with gut dysbiosis (Kwong and Moran 2016) and unhealthy colonies (Budge et al. 2016), but is negatively correlated with core microbiota members belonging to Lactobacillaceae, Orbaceae, and Neisseriaceae, 3 bacterial families known to contribute to the innate immune system of the honey bee (Kwong et al. 2017).

Beekeepers frequently treat their colonies with chemicals in fall to reduce the prevalence of parasites such as *Vairimorpha* sp.(microsporidian) and *Varroa destructor* (acarian). These parasites are known to negatively impact both the health of individual bees and colony survival (Fries 2009, Rosenkranz 2010). However, there is an increasing evidence suggesting that these molecules can affect negatively the honey bee health, and that most pathogens can develop a resistance to these treatments. For example, fumagilin, a common medication for nosemosis, alters honey bee midgut tissues at concentrations that do not eliminate *Vairimorpha* sp. (Huang et al. 2013). In some experiments, the quantity of *Vairimorpha* sp. spores was not reduced using fumagillin, but the survival of the bees improved (El-Khoury et al. 2018, Prouty et al. 2023). Moreover,

fumagillin, the active compound of Fumagilin-B, is restricted in many European countries due to its toxicity (van den Heever et al. 2014). Consequently, there is a need to develop safe and effective alternative treatments to ensure honey bee colony winter survival and spring development.

Probiotics are live microorganisms that, when administered in adequate amounts, confer a health benefit on the host (Hill et al. 2014). For example, under laboratory conditions, several probiotic strains of Lactobacillaceae and Bifidobacteriaceae can inhibit the growth of pathogenic bacteria, *Paenibacillus larvae* and *Melissococcus plutonius*, the causative agents of American and European foulbrood, respectively (Sabate et al. 2009, Forsgren et al. 2010, Killer et al. 2014, Janashia et al. 2016). In addition, trials conducted with caged honey bees showed that some probiotic bacterial strains provoke a reduction of the number of spores present in the gut of infected bees (Baffoni et al. 2016, Arredondo et al. 2018, Peghaire et al. 2020), but is it not always the case (Andrearcyzk et al. 2014, El-Khoury et al. 2018). Probiotic treatments can also mitigate the deleterious impacts of by improving honey bee survival, likely enhancing tolerance to the parasite (El-Khoury et al. 2018).

In field trials, colonies supplemented with lactic acid bacterium raised more brood (De Piano et al. 2017, Alberoni et al. 2018, Lyubimov et al. 2021), had a larger population (Audisio and Benítez-Ahrendts 2011, Daisley et al. 2023), and tended to produce more honey compared to nontreated colonies (Sabate et al. 2009, Audisio and Benítez-Ahrendts 2011, Patruica and Hutu 2013, Alberoni et al. 2018). The administration of Lactobacillus strains also reduces the *Paenibacillus larvae* pathogen loads in supplemented honey bee colonies, even in the presence of oxytetracycline, the medication used to treat American foulbrood (Daisley et al. 2021). Considering the benefits probiotics can have on honey bee health and colony performance, this alternative treatment has the potential to reduce the threat the overwintering period represents in northern regions, allowing colonies to thrive in spring.

Our goal was to assess the impacts of 3 probiotic formulas added to the fall feeding on the winter survival, spring development and *Vairimorpha* sp. spore load of honey bee colonies. One endogenous bacterium, *Bombella apis* (previously called *Parasaccharibacter apium*, hereafter *B. apis*) (Smith et al. 2021), and 2 commercial probiotic formulas (Bactocell and LevucellSB, Lallemand Inc.) were selected for this research.

Bombella apis is an endogenous bacterium that is mainly found in the gut of the queen bee and larvae, as well as in colony pollen bread and honey (Kwong and Moran 2016). Laboratory trials showed that this bacterium improves honey bee larval survival (Corby-Harris et al. 2014) and resistance to *V. Ceranae* (El Khoury et al. 2018). It also slightly increased colony winter survival (Corby-Harris et al. 2016).

Bactocell contains *Pediococcus acidilacti*, a bacterium that produces lactic acid, which regulates gut pH, and pediocin, an antibacterial compound (Di Giancamillo et al. 2008). This product is known to improve weight gain of the colonies and reduce pathogen load in honey bees (Castex 2009, Angelakis 2017). The bacterium *P. acidilacti* is naturally found in pollen collected by bees (Belhadj et al. 2010).

LevucellSB consists of *Saccharomyces cerevisiae boulardii*, a yeast used to reduce pathogen load in poultry (Rychen et al. 2017) and humans (Czerucka et al. 2007). In the beekeeping industry, *S. cerevisiae* is often used to enrich pollen substitutes (Kast & Roetschi, 2017).

Finally, Fumagilin-B antibiotic, which is the only medication authorized against *Vairimorpha* sp. in Canada (McCallum et al. 2020), was used in this study to compare both its effectiveness in mitigating nosemosis and its potential adverse effects on Spring brood production to that of the 3 probiotic formulas tested.

Materials and Methods

No animal health care permits were required for this research. The study took place at the Centre de Recherche en Sciences Animales de Deschambault (CRSAD, Deschambault, Québec, Canada; 46 40030.000 N, 71 54052.300 O) between September 2017 and June 2018. In mid-June 2017, 45 honey bee colonies were prepared with young sister queens, then placed in 2 honey-producing apiaries located on farmland near the research facility. At the beginning of

Table 1. Timeline of the manipulations and data collection during the experiment

2017				
Mid-June	September 12	October 12	October 18	November 5
45 colonies equally formed with sister queens	Colonies reduced to one brood chamber 24L 2: 1 sucrose feeding Thymovar (Varroa treatment) Varroa sampling *Vairispora* sp. sampling Broad area Hive weight	Probiotic treatment Fumagilin treatment Varroa sampling Hive weight	Probiotic treatment	Colonies moved to indoor wintering facility Varroa sampling *Vairispora* sp. sampling Hive weight Bee cluster size
Overwintering period (November, 2017 to April, 2018)				
2018				
April 20	**April 27**	**May 4**	**May 11**	**May 31**
Colonies moved to spring apiary Colony survival Hive weight Bee cluster size	Probiotic treatment *Vairispora* sp. sampling Varroa sampling	Probiotic treatment	Brood area Hive weight Varroa sampling	Brood area Hive weight Varroa sampling

September, honey supers were removed, and colonies were reduced to one brood chamber. Fall feeding started in mid-September and all colonies were given 24 liters of 2:1 sucrose solution using a top box feeder (Wooden Miller feeder # FE-1100 from Propolis-etc., Beloeil, QC, Canada). Colonies received a Thymovar anti-varroa treatment starting on September 12, followed by an oxalic acid treatment on November 5 (drip method: 35 g/L in a sucrose 1:1 solution, 5 ml between every frame of the hive body crowded with honey bees, to a maximum of 50 ml per colony). Colonies were wintered indoors in an environmentally controlled room (4–5 °C, 50–60% RH) from 22 November 2017, to 20 April 2018, and then moved to 2 spring apiaries until the end of June 2018.

Experimental Design

Prior to the experiment, the strength of the colonies was measured by visually estimating frame area covered with brood (Giovenazzo and Dubreuil 2011). Then, experimental groups were formed to have similar mean strength. The groups of 9 colonies were as follows: the first group (CTRL) was the control and received plain sucrose solution 1:1 (w/w); the second group (FMG) was treated with Fumagilin-B; the third group (PB1) was treated with the endogenous bacteria *B. apis*; the fourth (PB2) and fifth (PB3) groups were administered Bactocell and Levucell respectively.

Treatment Solutions and Their Administration

The *B. apis* strain was isolated from the gut of healthy workers in Québec, Canada, and characterized as described by El Khoury et al. (2018).The 16S RNA gene sequence of the *B. apis* strain used is ACA GTCAGATGTGAAATCCCCGGGCTTAACCTGGGAACTGCATT TGATACGTGCAGACTAGAGTCCGAGAGAGGGTTGTGGAATT CCCAGTGTAGAGGTGAAATTCGTAGATATTGGGAAGAACAC CGGTTGCGAAGGCGGCAACCTGGCTCGGAACTGACGCTGA GGCGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTG GTAGTCCACGCTGTAAACGATGTGTGCTGGATGTTGGGTG ATTTTATCATTCAGTGTCGGAGCTAACGCGTTAAGCACACC GCCTGGGGAGTACGGCCGCAAGGTTGAAACTCAAAGGAAT TGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAAT TCGAAGCAACGCGCAGAACCTTACCAGGGCTTGCATGGGG AGGCTGTATTCAGAGATGGATATTTCTTCGGACCTCCCGCA CAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATG TTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCTTTAGT TGCCATCACGTCTGGGTGGGCACTCTAGAGAGACTGCCGG TGACAAGCCGGAGGAAGGTGGGGATGACGTCAAGTCCTCA TGGCCCTTATGTCCTGGGCTACACACGTGCTACAATGGCGG TGACAGAGGGATGCTACATGGTACATGGTGCTGATCTCAAA AAACCGTCTCAGTTCGGATTGTACTCTGCAACTCGAGTGCA TGAAGGTGGAATCGCT. The sequence can be accessed through GeneBank, and the accession number is OR540530.

To initiate bacterial growth, an inoculum of the glycerol stock stored at −80 ℃ was streaked on Sabouraud Dextrose Agar (SDA) plates under sterile conditions and incubated aerobically at 37 °C for 48 h. Then, 3–4 colony-forming units (CFU) were transferred into 10 ml of liquid SDA medium and incubated under the same conditions as above. After 48 h, the broth was added to 1 liter of fresh SDA medium and incubated again at 37 °C on a rotary agitator. Every 24 h, the optical density of the broth was measured with a spectrophotometer, and the bacterial concentration was calculated with the standard curve. When the desired concentration was obtained, the bacterial broth was centrifuged at 4,000 RCF for 20 min and the supernatant was removed. The remaining bacterial pellet was divided and dissolved in nine 1 liter bottles of 1:1 sucrose solution the day prior to administration to ensure survival of the bacterias (El-Khoury et al. 2018).

The Bactocell and Levucell treatments were prepared dissolving the respective probiotic formula into 1:1 sucrose solution. The concentration of the 3 probiotic treatments was 10^9 CFU/L. probiotic solutions were prepared the day before administration and stored at 4 °C. Probiotic treatments PB1, PB2, and PB3 were given 4 times during the project: twice in fall (12 October and 18 October 2017) and twice in spring (27 April and 4 May 2018). For each treatment, colonies were given 1 liter of the prepared solution using a top box feeder (Wooden Miller feeder # FE-1100 from Propolis-etc., Beloeil, QC, Canada).

For the Fumagilin-B treatment (Group FMG), 9.08 g of the antibiotic was added to 1 liter of 1: 1 sucrose solution and given to each colony, as recommended by the manufacturer. This solution was prepared the day of administration, on 12 October 2017.

Colony Performance

To assess the impact of treatments on colony performance, we measured winter survival, brood area, weight variation, and bee population (Table 1).

***Winter survival*:**
Colony survival was noted in April when we moved the hives from the overwintering chamber to their spring apiary. A colony without brood or with less than 2 frames covered in bees was considered dead.

***Brood area*:**
The area occupied by immature worker honey bees (eggs + larva + sealed brood) in colonies was evaluated by measuring width and length of the brood area on each side of every brood frame. The rectangular area obtained was multiplied by 0.8 to compensate for the elliptic form of the brood pattern (Giovenazzo and Dubreuil 2011). These values were added to calculate the total brood area in each colony. A factor of 25 worker cells per 6.25 cm^2 (i.e., a square inch) was used to calculate the number of immature worker honey bees from the area. This calculation was first performed in September 2017 to create equal experimental groups, then twice in May 2018 (11 May and 1 June).

***Weight*:**
Hives were weighed monthly from September to November 2017, and from April to June 2018, using a numeric platform scale (total capacity of 500 kg, minimum weight sensitivity of 0.1 kg).

***Bee population*:**
The size of the bee cluster was measured in November and April, before and after the overwintering period. The number of frames covered by the cluster was noted, both from above and below, and the mean value was calculated for each colony.

Vairimorpha spp. Infection Level

Honey bees were sampled 3 times between September 2017 and April 2018: before the treatments (T0, 6 September 2017); after the fall treatments and before entering the wintering room (T1, 2 November 2017); and after removal from the wintering room (T2, 27 April 2018). Older worker honey bees were sampled to ensure the highest *Vairimorpha* sp infestation potential (Jack et al. 2016). These bees were sampled on side frames without brood and each sampling consisted of approximately 100 workers. Samples were immediately stored at −86 °C (Thermofisher −86 °C FORMA 908, Waltham, MA, USA).

Sixty worker bees per sample were pooled and the average spore load per bee was determined as described in (Cantwell 1970). Briefly, bees sampled were homogenized in 60 ml of 70% ethanol (1 ml/bee) in a Stomacher for 60 s at normal speed. One thousand microliters of the homogenate was transferred into a small tube for further microscopic examination using a hemocytometer under 400× magnification with a volume of 6 µL aliquot from the tube previously prepared. The *Vairimorpha* spores were identified and counted without segregating the species *V. ceranae* from *V. apis*.

V. destructor Infestation

Once a month, from September to November 2017 and from April to June 2018, the *V. destructor* mite population was monitored by placing sticky boards on the bottom board of each hive, covering the entire area. They were left in place for 7 consecutive days, and after removal, the daily mite drop was calculated. This variable was included in statistical analyses, as it is known to influence colony performance (Rosenkranz et al. 2010).

Statistical Analyses

Statistical analyses were conducted using R (v 3.3.1, Vienna, Austria), and P values < 0.05 were considered significant. To determine the impact of treatments on brood area and bee population, a mixed effect linear model was carried out. In each model, the fixed effects were the treatment and the varroa load, and the random effect was the apiary. The effects of the treatments on colony weight were determined using a linear model for repeated measures. The fixed effects were the time and the treatments, and the random effects were the colonies and the apiary. Finally, the impact of the treatments on *Vairimorpha* spp. spore load was assessed using a Negative Binomial mixed model for repeated measures that considered time and treatment as fixed effects, and the colonies as a random effect. For all measured variables, we compared treated groups to the control group with a Dunnett test.

Results

Colony Performance

During our study, one colony died during the overwintering period (FMG) and 2 died in spring (PB1 and PB3). These colonies were excluded from the analyses.

Sealed brood gain in spring was influenced by the experimental treatments given to the colonies (Fig. 1). The amount of sealed brood increased significantly in colonies treated with Bactocell (t = 2.50, P = 0.017) and Levucell (t = 2.72, P = 0.010), compared to control. Between May 11 and 31, colonies treated with Bactocell and Levucell gained respectively an average of 5,682 and 6,106 sealed brood cells, compared to 3,208 for the control colonies. The treatments did not impact the open and total brood gain in spring (Fig. 1).

From September 2017 to June 2018, the weight of the colonies from all treated groups was neither influenced by the antibiotic nor by the probiotic treatments (Fig. 2). Hives lost weight similarly

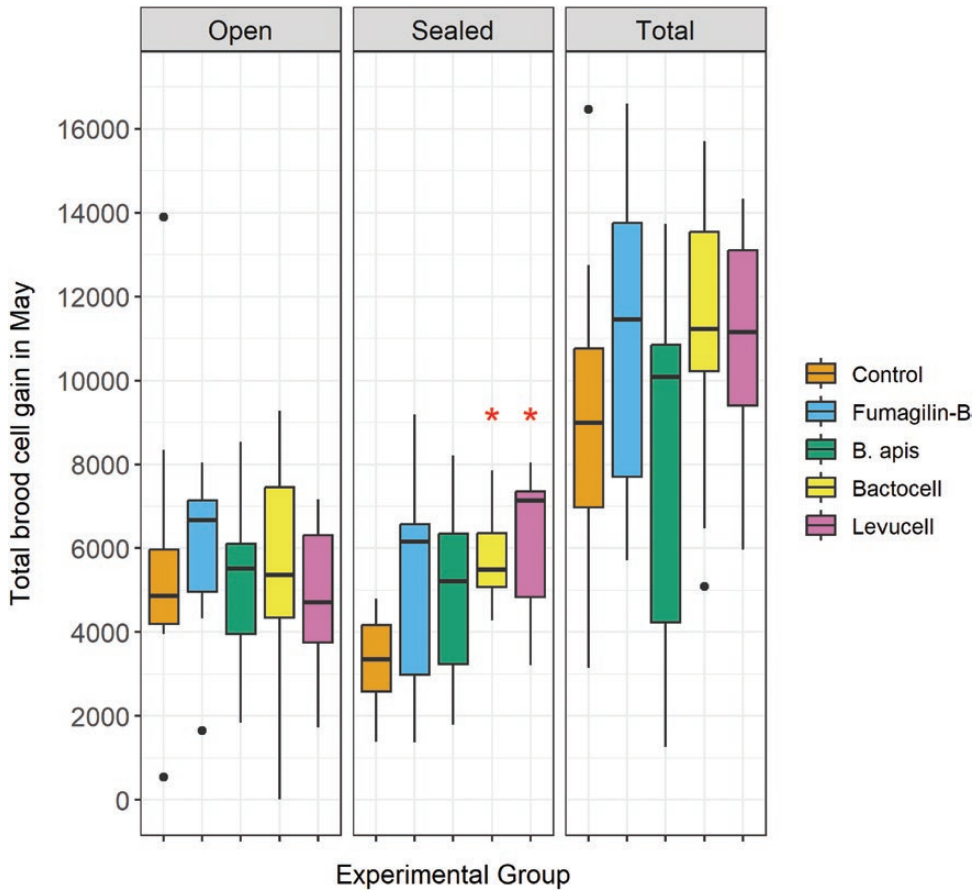

Fig. 1. Open, sealed and total brood cell gain between May 11 and 31, 2018 for each experimental group. Each group is compared to the control group. N = 9 colonies per group. * : $P \leq 0.05$.

during the overwintering period (from 22 November 2017 to 20 April 2018).

There was no significant difference in the number of frames covered in bees between the treated groups and the control group either in November, before the overwintering period, or in April, when they were moved from the indoor facility to the apiary (Fig. 3).

Vairimorpha spp. Infection Level

At the beginning of the experiment, the *Vairimorpha* spp. spore load was low for all groups and there was no difference between them (Fig. 4). The only treatment that significantly reduced the spore load in spring was Fumagilin-B ($Z = -2.22$, $P = 0.027$). The groups treated with probiotics showed similar spore loads to the control group throughout the entire experiment.

Discussion and Conclusion

The goal of this study was to assess the effects of 3 probiotic treatments on honey bee colony winter survival, spring performance, and *Vairimorpha spp*. spore load. Colonies treated with the commercial probiotic formulas Bactocell and Levucell gained respectively 77% and 90% more sealed brood in spring than the control group colonies. Some authors suggest that colonies treated with probiotics produce more brood because the treatment stimulates egg laying (Audisio and Benítez-Ahrendts 2011, Sabate et al. 2012). However, this hypothesis is not supported by any mechanism identified to date.

In a healthy colony, 85% of laid eggs will develop into adults, compared to 64% in a weak colony (Fukuda and Sakagami 1968). The increase in sealed brood observed in our experiment suggests that larval survival rate was higher in colonies supplemented with probiotics. A first factor that may have improved larval survival was increased food availability and quality. In a trial conducted in bumble bee (*Bombus terrestris*) microcolonies and using *Lactobacillus kunkeei* as a probiotic (now reclassified as *Apilactobacillus kunkeei* (Zheng et al. 2020)), the bee population increased in treated colonies when available food was of low quality (Billiet et al. 2017). The authors hypothesized that A. *kunkeei* increased food digestion and assimilation, thus allowing these colonies to thrive and raise more larvae. Poorly fed colonies were observed to reduce larvae production instead of raising poor quality offspring (Torres et al. 2015). Since food sources are scarce in fall and spring in Québec, it is plausible that colonies supplemented with Bactocell and Levucell were able to maximize nutrients from the foraged pollen and nectar, consequently increasing brood rearing and reducing larval mortality. It would be relevant to assess further the impact of both probiotic strains on nutrition in controlled caged trials, in order to measure accurately survival and body fat percentage, for instance.

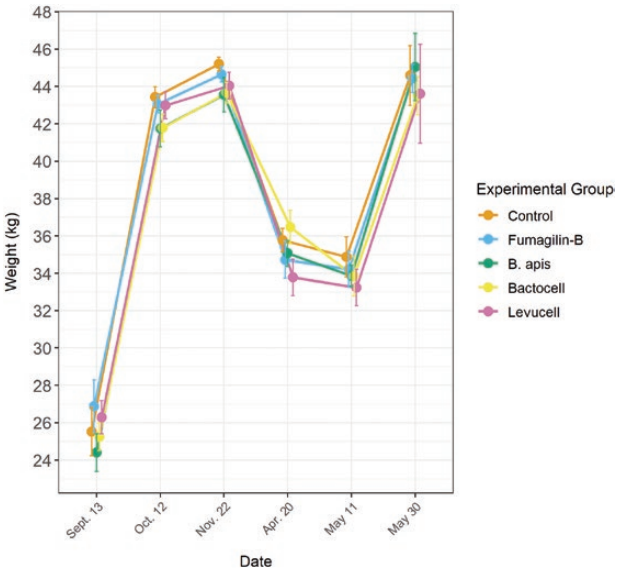

Fig. 2. Average weight of the colonies during the experiment. Weight was measured once a month, except during the overwintering period (December to March). Error bars indicate standard error. N = 9 colonies per group. No significant differences between treatments were detected.

Secondly, many researchers have shown that endogenic and commercial strains of *Lactobacillus sp.* enhance colony performance by increasing the worker population and honey production (Audisio and Benítez-Ahrendts 2011, Sabate et al. 2012, Patruica and Hutu 2013, Audisio et al. 2015, Alberoni et al. 2018, Khaled et al. 2018). In our study, no impact on hive weight was observed between September and June. However, since honey is mainly harvested from June to September in Québec, it is possible that treated colonies with more sealed brood would have a larger population later in summer, harvest more honey and be better prepared to survive the following winter. A long-term study of the impact of Bactocell and Levucell as a preventive treatment in summer would test this hypothesis.

To reduce winter mortality and promote colony growth, many beekeepers prevent nosemosis using fumagillin. Our results confirm the efficacy of this treatment in reducing *Vairimorpha* spp. cells in treated colonies. Despite its efficacy, *Vairimorpha* spp. could develop resistance to fumagillin in the coming years, as has been observed in several microorganisms (Tyers and Wright 2019). Furthermore, it was shown that fumagillin must be used with caution: when exposed to a low concentration of the product, *Vairimorpha spp*. spore production increases, while the honey bee gut epithelial barrier is disrupted (Huang et al. 2013). It is therefore essential to explore the potential of probiotics as an effective and safe alternative to conventional treatments.

In our experiment, although no significant impact of our probiotic treatments on *Vairimorpha* spp. spore load was detected, *B. apis* and Levucell showed a trend in reducing spore per bee (Fig. 4). During caged trials, it was shown that bees supplemented with *B. apis* (called *P. apium*) have a lower *Vairimorpha spp*. spore load than nontreated bees (Corby-Harris et al. 2016). El Khoury et al. (2018) also noted that this endogenic bacterium, Bactocell and Levucell improved honey bee survival when infected with *Vairimorpha spp*. In their experiment, treated bees were found to have the same spore load as nontreated bees, but their survival rate was significantly higher. This tolerance to the presence of *Vairimorpha spp*. could be explained by the fact that certain probiotics can protect the gut epithelium (Oelschlaeger 2010) and stimulate the immune response of the bee (Janashia et al. 2016). Interestingly, in the present *in situ* trial, 2 out of 3 probiotic strains (*B. apis* and Levucell) showed a trend to reduce spore loads in bees but it was not significant. Therefore, administration of higher probiotic concentrations, adding treatments during the overwintering period or alternate ways of delivering the probiotics (Daisley et al. 2023) should be undertaken to validate their potential efficiency in reducing spore loads. Here, we settled on a concentration of 10^9 CFU/L based on previous in situ experiments (Audisio et al. 2015, Alberoni et al. 2018). However, considering that the hive environment may decrease probiotic survival, it could be interesting to increase either treatment concentration, frequency of delivery mechanism to ensure its effectiveness.

Overall, our results support current knowledge concerning the benefits of using probiotics to enhance performance of honey bee

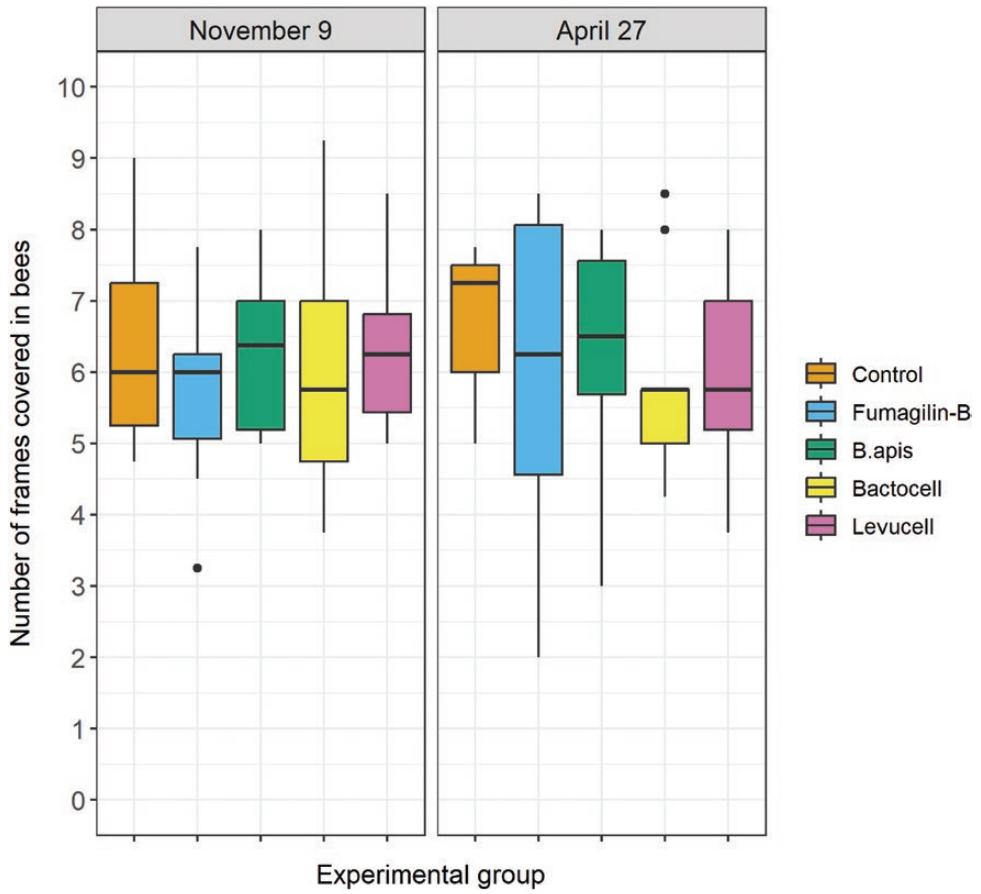

Fig. 3. Number of frames covered in bees for each experimental group, in fall (November 9, 2017) and spring (April 9, 2018). N = 9 colonies per group. No significant differences between treatments were detected.

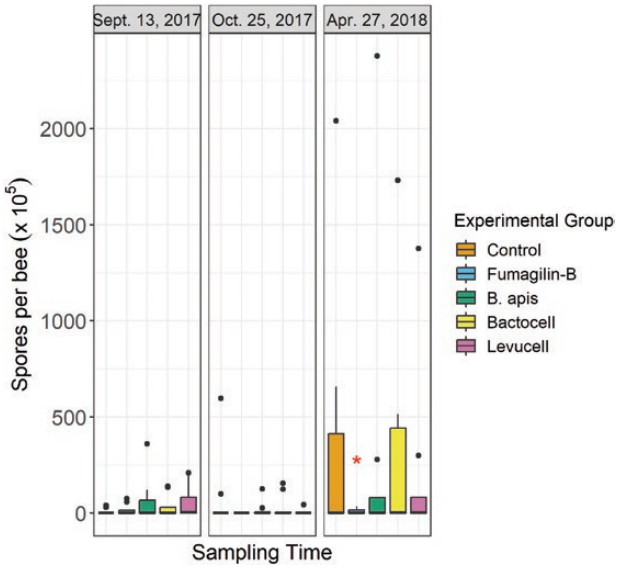

Fig. 4. Number of *Vairimorpha spp.* spores per bee at three sampling times. Each group is compared to the control group. * : $P \leq 0.05$.

colonies. In this experiment, 3 single strains of beneficial bacteria were tested on honey bee colonies during the overwintering period. Colonies treated with Bactocell and Levucell showed a significant increase in sealed brood in spring. Our results show that probiotic treatments can play an important role in the beekeeping industry due to their beneficial impact on honey bee health. Additional experiments are needed to determine the optimal concentration, administration frequency, and delivery method for probiotic formulas to become a highly effective treatment option for the beekeeping industry.

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Author Contributions

Naomie Bleau (Conceptualization [Equal], Data curation [Equal], Formal analysis [Equal], Investigation [Equal], Methodology [Equal], Software [Equal], Visualization [Equal], Writing – original draft [Equal], Writing – review & editing [Equal]), Nicolas Derome (Conceptualization [Equal], Methodology [Equal], Project administration [Equal], Resources [Equal], Supervision [Equal], Validation [Equal], Writing – review & editing [Equal]), and pierre giovenazzo (Conceptualization [Equal], Funding acquisition [Equal], Methodology [Equal], Project administration [Equal], Resources [Equal], Supervision [Equal], Validation [Equal], Writing – review & editing [Equal])

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