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# The dose makes the poison: feeding of antibiotic-treated winter honey bees, *Apis mellifera*, with probiotics and b-vitamins

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**Abstract** – Honey stores of *Apis mellifera* colonies are replaced with sugar water by beekeepers, which may result in malnutrition. Nutritional supplements have been developed, but the importance of bacterial probiotics and vitamins is poorly understood. Given that supplementary feeding with vitamins and probiotics may enhance worker weight and longevity, this might suggest a feasible approach to mitigate winter colony losses. Here, we conducted a laboratory hoarding cage study with freshly emerged winter bees, which were treated with the antibiotic tetracycline to reduce gut bacteria obtained post-emergence and subsequently assigned to feeding regimes: sucrose only, sucrose + pollen, probiotics (low and high dosage), probiotics + pollen (low and high dosage), or b-vitamins (low and high dosage) ( $N=8$  treatments, 29 workers/cage  $\times$  8 replicates). In parallel, another age cohort of bees remained on their frame (=Frame) to establish their gut microbiota and were subsequently fed with sucrose only or sucrose + pollen ( $N=2$  treatments, 29 workers/cage  $\times$  4 replicates). The most beneficial effects on body weights were found in workers given *ad libitum* access to pollen, notably in the Frame Sucrose + Pollen group, confirming the inherent importance of post-emergent gut flora inoculation and the role of gut bacteria in protein digestion. Furthermore, both Frame groups and the antibiotic-treated workers fed with probiotic low + pollen survived longer than all other groups, highlighting a fundamental host-microbial relationship. On the other hand, our current treatments alone, post-tetracycline, did not yield any positive results. In contrast, high dosages of both probiotic and b-vitamins significantly reduced lifespan compared to their low concentration counterparts, probably due to dysbiosis and toxicity, suggesting that the outcome was dose-dependent. These results highlight that bacterial and b-vitamin supplementation can alter longevity with advisable caution since harmful concentrations are possible to exist.

*Apis mellifera* / Nutrition / Gut microbiota / b-vitamins

## 1. INTRODUCTION

Adequate nutrition is paramount for honey bee, *Apis mellifera*, colony survival (reviewed in Brodschneider & Crailsheim, 2010). Recent

winter colony losses have brought immense awareness to apiculture (Neumann & Carreck, 2010) and inadequate nutrition has been identified as one of several key indicators for hive failure (reviewed in Brodschneider et al., 2010; van Engelsdorp et al., 2010). Various reasons explain why a colony might lack proper nutrition, such as reduced availability to forage due to altered landscapes from agriculture (Naug, 2009)

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or over intense honey harvesting from beekeepers (reviewed in Brodschneider & Crailsheim, 2010). In order to sustain colony development, honey bees forage pollen and nectar from spring to autumn flower efflorescence (Klein et al., 2019) and store the food in the forms of bee bread and honey for the times of reduced foraging, i.e., winter (Winston, 1991). Traditionally, beekeepers remove honey stores and replace it with simple sucrose solution, yet often times, micronutrients such as vitamins are wholly neglected in the supplementing process of adult workers. If an over-intense honey harvest occurs, replaced solely with sucrose solution, this may result in low micronutrient dense energy stores for the critical over-wintering time.

Substantial *A. mellifera* nutritional research has demonstrated the importance of pollen (Frias et al., 2016), showing its vital ties between nurse and emergent bees (Haydak, 1935), as well as causal associations with honey bee health proxies, namely, longevity (Maurizio, 1954) and body weight (Retschnig et al., 2021). Fortunately, decade's long in-depth reflection has been dedicated to the nutritional requirements of honey bees, with a particular emphasis on macronutrients (reviewed in Brodschneider & Crailsheim, 2010; reviewed in Pudasaini et al., 2020). In line with such research, evidence-based macronutrient supplementation practices are readily available for beekeepers to consult (Standifer et al., 1977), and commercially made products are available (Somerville, 2000). On the other hand, gaps in the present understanding of the micronutritional needs (i.e., vitamins) of adult honey bees persist.

To date, little work has been done that focuses on a branch of micronutrients, b-vitamins, and their requirements for *A. mellifera* workers (reviewed in Brodschneider & Crailsheim, 2010). Insects are predominantly unable to synthesize eight essential b-vitamins themselves (Douglas, 2017), but have been reported to require them for metabolic processes like fatty acid synthesis (biotin, vitamin B7) or nucleic acid biosynthesis (folic acid, vitamin B9) (Dadd, 1973). In *A. mellifera*, pyridoxine (vitamin B6) is necessary for brood rearing (Haydak & Dietz, 1972) and measurable doses of required pyridoxine needed

for brood rearing have already been published (Anderson & Dietz, 1976). Furthermore, recent data suggest that b-vitamins positively contribute to colony performance (Jovanovic et al., 2021). Insects need a steady supply of b-vitamins, given their limited capacity of being able to store them (Douglas, 2017), and typically, they obtain them from a combination of their external environment as well as host-microbial symbionts, otherwise referred to as their gut microbiome (Salem et al., 2014).

Nutrition-based interactions with a host's microbiota are well established in animals (Douglas, 1998; Duncan et al., 2008), and animal health is considerably intertwined with the bacterial community within the digestive tract (Martinson et al., 2012). This mutualistic host-microorganism relationship in *A. mellifera* workers includes vitamin production (Kwong et al., 2014), as well as plant secondary metabolite detoxification (Kešnerová et al., 2017), immune function support (Tejerina et al., 2021; Vásquez et al., 2012), and integral links to longevity (Raymann et al., 2017). Emergent bees are born sterile and are fully inoculated with their core microbiota shortly after emergence, primarily from physical contact with the nest down to social interactions with nestmates (Martinson et al., 2012), forming a core microbiota composed primarily of lactobacilli that, if left undisturbed, are highly conserved throughout a worker bee's lifespan (Kwong & Moran, 2016). Given their apparent beneficial effects, *Lactobacillus* supplementation (i.e., probiotics) to honey bees may prolong lifespan thereby providing a feasible gateway to improving winter colony survival.

At present, b-vitamin and probiotic supplementation lack concrete quantitative and qualitative data for *A. mellifera*. B-vitamins have been labeled as generally safe for insects even if administered at high doses (Douglas, 2017); however, habitual exposure to elevated levels of b-vitamins has shown to be harmful in other taxa (Phillips et al., 1978). Furthermore, even if certain strains of probiotics are beneficial, a possible reversal of benefits from probiotics can occur in an overgrowth scenario (Hayashi et al., 2017). Both of these aspects highlight

the relevant importance of proper dosing. Furthermore, in order to better isolate b-vitamin dosing, decoupling innate b-vitamin synthesis from *A. mellifera* microbiomes is also necessary a step to consider. Therefore, the present study aims to undertake hoarding cage experiments to explore if b-vitamins and probiotics administered at different dosages influence antibiotic-treated winter worker bee longevity and body weights, with the goal of uncovering a starting point for micronutrient and probiotic supplementation. We would expect to see an improvement on the measured parameters as a result from supplementation.

## 2. MATERIAL AND METHODS

### 2.1. Experimental setup

The experiment was conducted from October 22<sup>nd</sup> to December 31<sup>st</sup> 2019 (local autumn and winter seasons) at the Institute of Bee Health in Bern, Switzerland. To obtain defined age cohorts of newly emerged workers, in addition to isolating late autumn brood to ensure long-lived winter workers, queens from local *A. mellifera* colonies ( $N=5$ ) were caged onto open drawn comb, and after 20 days, capped worker brood frames

were incubated until adult emergence (48 h, 34.5 °C, > 60% RH) (Williams et al., 2013). After 48 h, newly emerged workers from all colonies were mixed, standardizing the impact of genetics between colonies, and randomly placed in hoarding cages ( $N=64$  cages, 29 bees/cage,  $N=$ total workers 1856) (Williams et al., 2013). After being completely cleared of emergent bees, the same frames were incubated again, and 24 h later, a second group of freshly emerged workers were marked individually (UniPosca color tip pens), put back on their original frame, and incubated at 30 °C and > 60% RH (Williams et al., 2013) for 48 additional hours, resulting in 72-h old worker bees. Afterwards, the workers were removed and also mixed in a single container and assigned to cages ( $N=8$ , 29 bees/cage, total workers  $N=232$ ). The first group of workers were treated with tetracycline for 72 h to reduce gut microbiota and then assigned to one out of eight treatments (Table I, groups 3–10), while in parallel to the antibiotic treatment, workers from the 2nd incubation remained on their frames, retaining access to nutrients and bacteria, and were designated to one out of two feeding regimens (Table I, groups 1–2, hereafter referred to as “Frame” bees). All workers were maintained at 30 °C and > 60% RH (Williams et al., 2013) until the last worker died.

**Table I** Treatment groups of *Apis mellifera* workers from the hoarding cage trial. Groups 1–2:  $N=4$  cages per treatment, 29 workers/cage,  $N=116$  workers per treatment, total  $N=232$ . Groups 3–10:  $N=8$  cages per treatment, 29 workers/cage,  $N=232$  workers per treatment, total  $N=1856$

| Group | Treatment               | Tetracycline | Frame | Pollen | Probiotics              | Vitamins | Sample size |
|-------|-------------------------|--------------|-------|--------|-------------------------|----------|-------------|
| 1     | Frame Sucrose           | –            | +     | –      | –                       | –        | 116         |
| 2     | Frame Sucrose + Pollen  | –            | +     | +      | –                       | –        | 116         |
| 3     | Sucrose                 | +            | –     | –      | –                       | –        | 232         |
| 4     | Sucrose + Pollen        | +            | –     | +      | –                       | –        | 232         |
| 5     | Vitamin Low             | +            | –     | –      | –                       | 300 mg/L | 232         |
| 6     | Vitamin High            | +            | –     | –      | –                       | 9 g/L    | 232         |
| 7     | Probiotic Low           | +            | –     | –      | $4.1 \times 10^6$ CFU/L | –        | 232         |
| 8     | Probiotic High          | +            | –     | –      | $4.1 \times 10^7$ CFU/L | –        | 232         |
| 9     | Probiotic Low + Pollen  | +            | –     | +      | $4.1 \times 10^6$ CFU/L | –        | 232         |
| 10    | Probiotic High + Pollen | +            | –     | +      | $4.1 \times 10^7$ CFU/L | –        | 232         |

## 2.2. Treatments

### 2.2.1. Antibiotic

Tetracycline hydrochloride (©Sigma-Aldrich) was assessed in two hoarding cage pre-trials, first with freshly emerged winter bees and later with freshly emerged summer bees, and was found as the best suitable substance to inactivate the gut microbiota without significantly influencing longevity (Figs. 3 and 4). Following those results for this trial, a tetracycline-sucrose solution (50% (w/v), 500 µg/mL tetracycline hydrochloride) was prepared and supplied *ad libitum* to the antibiotic-treated groups for 72 h.

### 2.2.2. Dietary treatments

Sucrose solution (50% w/v) made with sterilized tap water was mixed with either vitamins, probiotics, or left blank (control), and fed to all workers *ad libitum* (Table I). All treatment solutions were prepared freshly on a weekly basis and stored at 4 °C. Four treatment groups (2, 4, 9, and 10) were additionally fed *ad libitum* non-sterilized corbicula pollen from honey bees (Swiss Pollen, Bienen Roth). The pollen was stored at -24 °C, and prior to feeding, was thawed, ground, and packed into 1.5-mL microcentrifuge tubes with a clipped tip and supplied to workers. Syringes and pollen tubes

were changed with fresh sterilized ones on a bi-weekly basis.

### 2.2.3. B-vitamins

Two b-vitamin dosages were used (1) Vitamin Low (300 mg/L) and (2) Vitamin High (9000 mg/L). The Vitamin Low dosage was chosen based on b-vitamin estimates in the natural diet (as reviewed in Bogdanov et al., 2008) and daily food consumption (Winston 1991), and the high dosage was chosen to explore possible toxic effects (Douglas, 2017). Pure vitamin isolates were purchased from Hänseler AG (B1, B2, B3, B5, B6, B8), PureBulk (B9), and Sanofi Chimie BP (B12). Stock concentrations were made by solving each vitamin in deionized water, then adding those to a 50% (w/v) sucrose solution resulting in two mixes (Table II).

### 2.2.4. Probiotics

The pharmaceutical product Lactibiane Voyage, which contains a mix of *Lactobacillus acidophilus* (strain LA 201), *Lactobacillus plantarum* (strain LA 301), and *Lactobacillus casei* (strain LA 205), was used to make two probiotic dosages: (1) Probiotic Low:  $4.1 \times 10^6$  CFU/L and (2) Probiotic High:  $4.1 \times 10^7$  CFU/L, similar to total viable CFUs in *A. mellifera* honey crops

**Table II** Quantity of individual b-vitamins supplement to *Apis mellifera* workers in the treatments: (1) Vitamin Low and (2) Vitamin High. Proportion in % rounded to the 100th digit

| B-vitamin        |       | Vitamin Low (mg/L) | Vitamin High (mg/L) | Proportion (%) |
|------------------|-------|--------------------|---------------------|----------------|
| Thiamine         | (B1)  | 10                 | 300                 | 3.29           |
| Riboflavin       | (B2)  | 20                 | 600                 | 6.58           |
| Nicotinamide     | (B3)  | 200                | 6000                | 65.83          |
| Pantothenic acid | (B5)  | 50                 | 1500                | 16.46          |
| Pyridoxine       | (B6)  | 20                 | 600                 | 6.58           |
| Biotin           | (B8)  | 0.75               | 22.5                | 0.25           |
| Folic acid       | (B9)  | 3                  | 90                  | 0.99           |
| Cyanocobalamin   | (B12) | 0.0375             | 1.125               | 0.01           |
| Total            | 8     | 303.8              | 9113.63             | 100            |

(Vásquez et al., 2012). The probiotics were dissolved in deionized water and directly mixed with 50% (w/v) sucrose solution to obtain the final dosages.

To confirm that the bacteria were alive throughout the treatment process, the presence of *Lactobacillus* bacteria was confirmed by culture on *Lactobacillus* specific MRS agar plates and identification was made using matrix-assisted-laser-desorption/ionization-time-of-flight-mass-spectrometry (MALDI-TOF MS) (Microflex LT, Bruker, Daltonics) using the Biotyper software with Bruker MBT 7854 MSP Library. Species detection was performed using default parameters and identification was confirmed when the identification score was  $\geq 2.0$ . Samples were taken from live workers on days 10 and 20 from the treatments: (1) Sucrose + Pollen, (2) Probiotic High, and (3) Probiotic High + Pollen ( $N=5$  workers/treatment/day). Samples were dissected following steps from Carreck et al. (2013), and cultured on *Lactobacillus* specific MRS agar (© Sigma-Aldrich) plates and incubated for 48 h at 37 °C under anaerobic conditions.

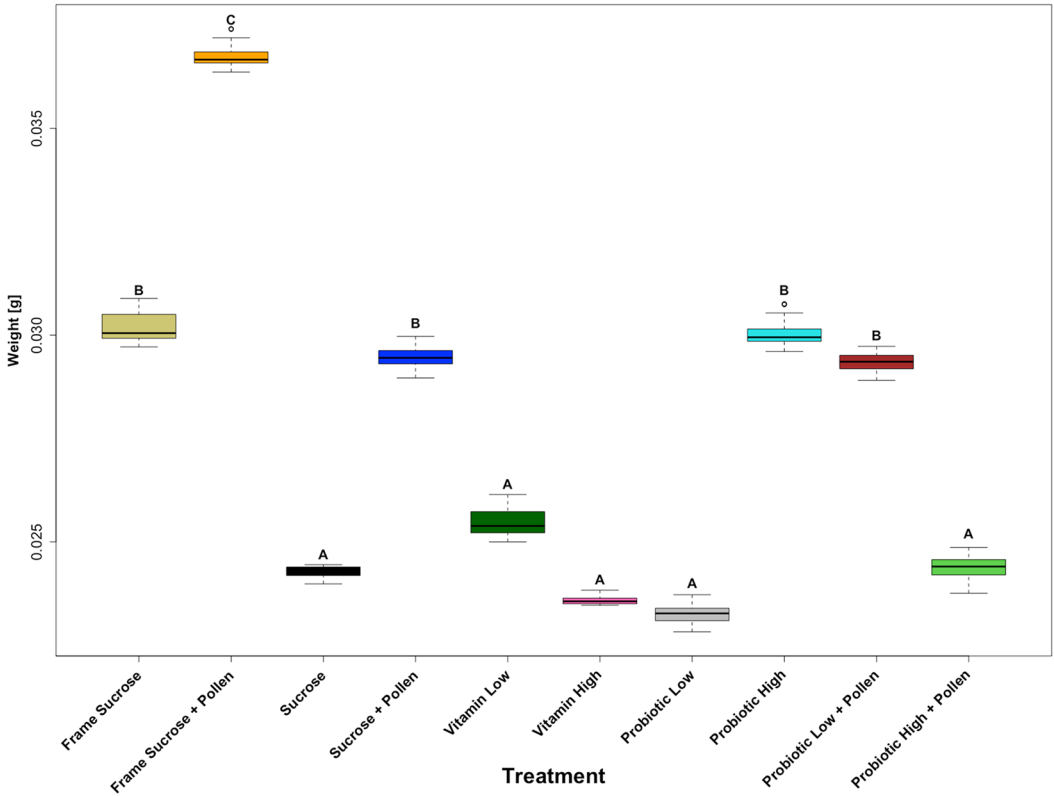
### 2.3. Mortality, sucrose consumption, and body weight

Mortality and sucrose-solution consumption were recorded daily. Dead workers were removed and stored at  $-80$  °C. To estimate consumption of sucrose solution per worker per day, the syringes were weighed daily and the differences in weight were divided by the number of live workers present in the cage at that time. To account for evaporation from the incubator, ten control syringes were filled with sucrose solution and put in cages without workers, incubated, and measured daily, and the average evaporation rates were used to adjust sucrose-solution consumption across all treatments (OECD, 2017). Due to treatment-induced mortality, individual body weights of up to three live workers per cage were measured when possible on days 7, 14, and 21 (Supplement Table S1.1) using a Mettler AT 400 scale with a precision range of  $10^{-4}$  g. From these workers, up to two were subsampled when

possible from each treatment group and each day (Supplement Table S2.1) and dried to assess dry body weight (Brodschneider et al., 2009). To do so, samples were stored in liquid nitrogen, transferred into a Zirbus Sublimator VaCO 2 (Bad Grund, Germany), lyophilized, re-weighed using the same Mettler AT 400 scale, and repeated until constant dry weights (Table S2.1).

### 2.4. Statistical analyses

Statistical analyses were performed using the program, R, version 3.6.2 (R Core Team, 2008). For survival analysis, the packages “survival” (Therneau, 2021; Therneau & Grambsch, 2000) and “surminer” (Kosinski et al., 2020) were used to calculate and plot Kaplan–Meier survival curves. The `Survdiff` function was used to calculate survival curves and log rank testing ( $\rho=0$ ) as well as to perform a chi-squared test. The `pairwise_survdif` function was used for multiple comparisons from the survival analysis between all treatment groups, and the resulting  $p$  values were adjusted for multiple comparisons using a Bonferroni method (Bonferroni, 1936). All data for the analysis of the body weight and sucrose consumption were log-transformed and used in linear mixed effect models (`lmer`), using the R package “lme4” (Bates et al., 2015) and fitted using “REML [`lmerMod`]”, while defining “cage” and “day” as random factors. Histograms and qqPlots of the model residuals were used to verify model assumptions. Post hoc `lmer` pairwise testing was done on the sucrose consumption and body weight data using the “multcomp” package (Hothorn et al., 2008), selecting “Tukey” comparison of means, with “Holm” correction (Holm, 1979) from the extracted fitted values of the model. To obtain the compact letter display (Piepho, 2004) used in Figs. 1 and 2, the function `cld()` was used from the “multcomp” package (Hothorn et al., 2008), and the function `multcompLetters()` from the `multcompView` package (Graves et al., 2019) for Figs. 3, 4, and 5. Finally, additional linear mixed effect models were carried out for the body weight and sucrose consumption data, with the explanatory



**Figure 1.** Boxplots of the predicted dry body weight values of workers, *Apis mellifera*, from 10 treatment groups, extracted from a linear mixed effect model (lmer) which was calculated using the log-transformed dry body weight of the workers, dependent on diet (treatment), and “cage” and “day” as random variables ( $N=10$  treatments,  $N=1-16$  workers per treatment, total  $N=356$  total workers). Post hoc testing from the lmer, comparing group means (Tukey), with Holm correction was used to determine statistical significance. Compact letter display indicates statistical difference between treatments ( $ps < 0.05$ ).

variables (i.e., treatments) expressed as indicator variables (excluding “frame”), with “cage” and “day” defined as random factors, to estimate the effects of the explanatory variables as well as to test for any significant interactions.

### 3. RESULTS

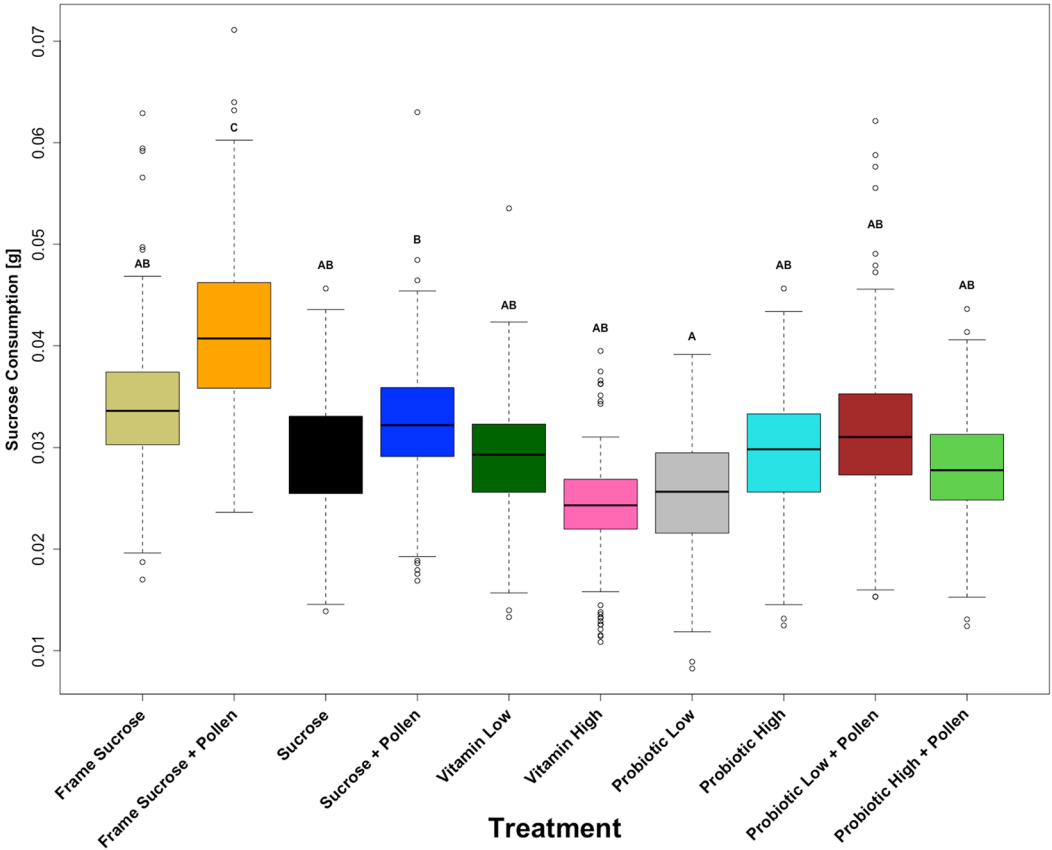
#### 3.1. Probiotics treatment confirmation

The *Lactobacillus* bacteria fed were found in the mid- and hindguts of workers, confirming a successful probiotic feeding method. Samples from the group “Sucrose + Pollen” yielded

negative results from both days 10 and 20 of sampling, not growing any *Lactobacillus* containing colonies on the agar plates. However, on both sample days from the groups “Probiotic High” and “Probiotic High + Pollen,” live bacteria from the supplemented species *Lactobacillus acidophilus* (strain LA 201), *Lactobacillus plantarum* (strain LA 301), and *Lactobacillus casei* (strain LA 205) were successfully detected.

#### 3.2. Body weight

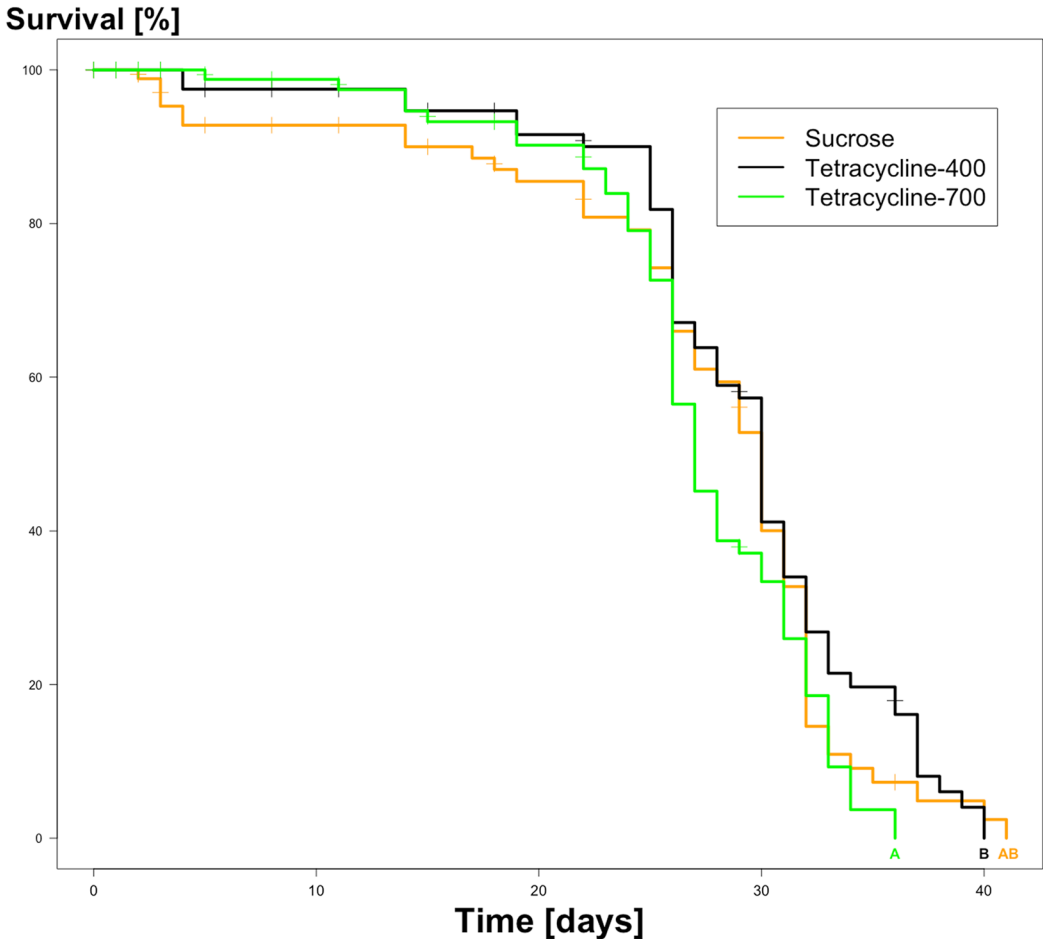
The dry body weight of all experimental workers ranged from 0.0152 to 0.0565 g ( $N=1-16$



**Figure 2.** Boxplots of the fitted daily sucrose solution consumption values of workers, *Apis mellifera*, per worker/per cage from 10 treatment groups, extracted from a linear mixed effect model (lmer) calculated with the log-transformed consumption rates of the workers, dependent on diet (treatment), and “cage” and “day” as random variables ( $N=10$  treatments,  $N=4-8$  cages per treatment,  $N=2232$  total measurements). Post hoc testing from the lmer, comparing group means (Tukey), with Holm correction was used to determine statistical significance. Compact letter display indicates statistical difference between treatments ( $ps < 0.05$ ).

workers per treatment, total  $N=356$ , Table S2.1). Exploratory analysis showed significant variation caused by our treatments (analysis of variance (ANOVA) of linear mixed effect model (lmer) ( $F(9,346) = 13.4, p < 0.001$ )). Post hoc testing on the fitted (i.e., predicted) values extracted from the lmer (Fig. 1) revealed three distinct groups (lmer, Tukey multiple mean comparison, Holm adjusted  $p$  values, all  $ps < 0.05$ ). Highest body weights were detected in the group “Frame Sucrose + Pollen” (Fig. 1, letter C), followed by the “Frame Sucrose,” “Sucrose + Pollen,” “Probiotic High,” and “Probiotic Low + Pollen”

groups (Fig. 1, letter B). The lowest body weights were recorded in the groups “Sucrose,” “Vitamin Low,” “Vitamin High,” “Probiotic Low,” and “Probiotic High + Pollen” (Fig. 1, letter A). Furthermore, an additional multiple regression model ran with indicator variables, excluding “Frame,” revealed that pollen had a positive influence on body weight (lmer, coefficient = 0.0056,  $p < 0.001$ ) as well as high levels of probiotics (lmer, coefficient = 0.0035,  $p = 0.03$ ), low levels of probiotics negatively influenced body weight (lmer, coefficient = -0.0033,  $p = 0.04$ ) and lastly, that there was a significant negative interaction



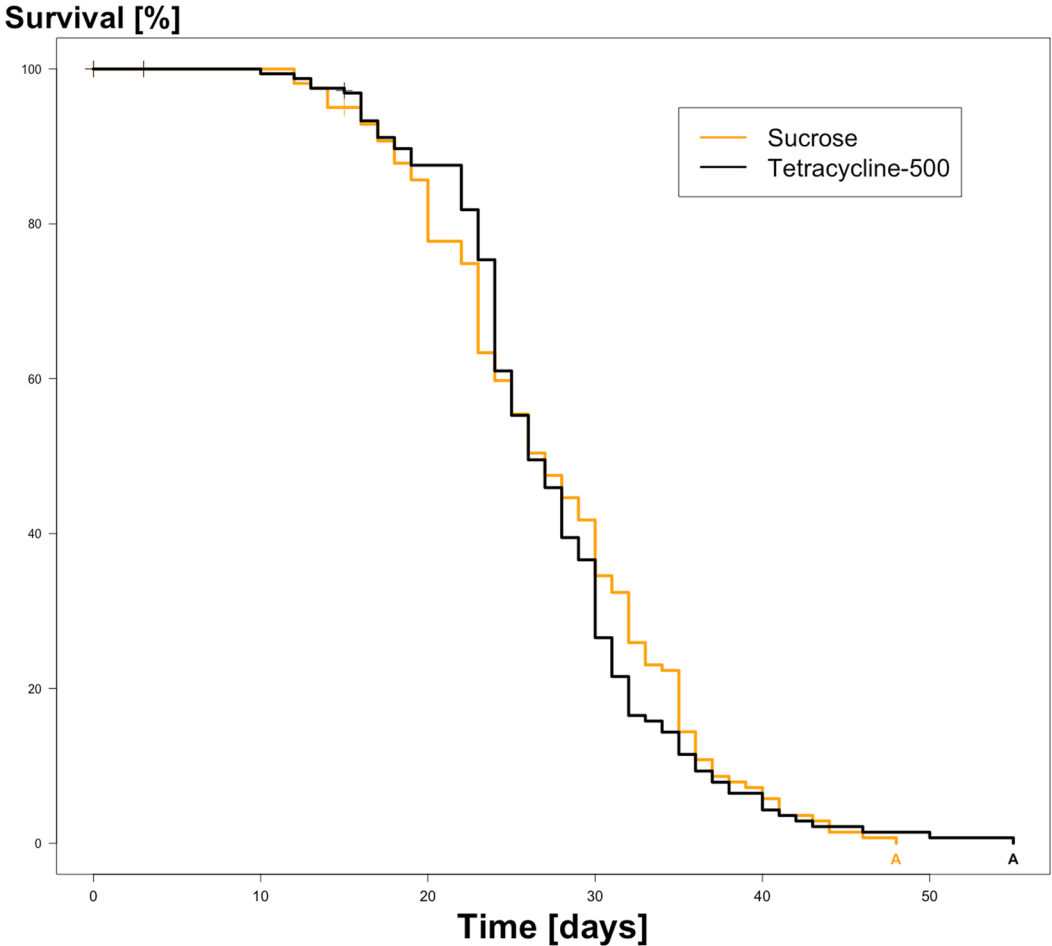
**Figure 3.** Kaplan–Meier survival curves of three groups of experimental winter workers, two of which were treated with tetracycline at dosages of 400 µg/mL or 700 µg/mL (Tetracycline-400 and Tetracycline-700) supplied in sucrose solution (50%, w/v) and a control supplied with sucrose solution only (50%, w/v).  $N=90$  workers per treatment group (3 cages/group with 30 bees each).  $N=270$  total workers. Significant differences between the survival of the experimental workers are indicated by compact letter display based on log rank tests and Bonferroni  $p$  adjusted values ( $ps < 0.05$ ).

between the two categories “Probiotic High” and “Pollen” resulting in significant reduction in body weight (lmer, coefficient = 0.0105,  $p < 0.001$ ).

### 3.3. Sucrose consumption

Sucrose solution consumption data were used to calculate an average consumption rate per worker/per cage/per day ( $N=4-8$  cages per treatment,  $N=2232$  total measurements).

Consumption rate values ranged from 0.00 to 0.137 g. Control cages without workers showed  $< 0.01\%$  evaporation due to the incubators, and all consumption values were adjusted accordingly to account for evaporation. Exploratory analysis showed significant variation caused by the treatments (analysis of variance (ANOVA) of linear mixed effect model (lmer) ( $F(9,2222) = 18.39, p < 0.000$ )). Post hoc testing on the fitted values from the lmer (lmer, Tukey multiple mean comparison,



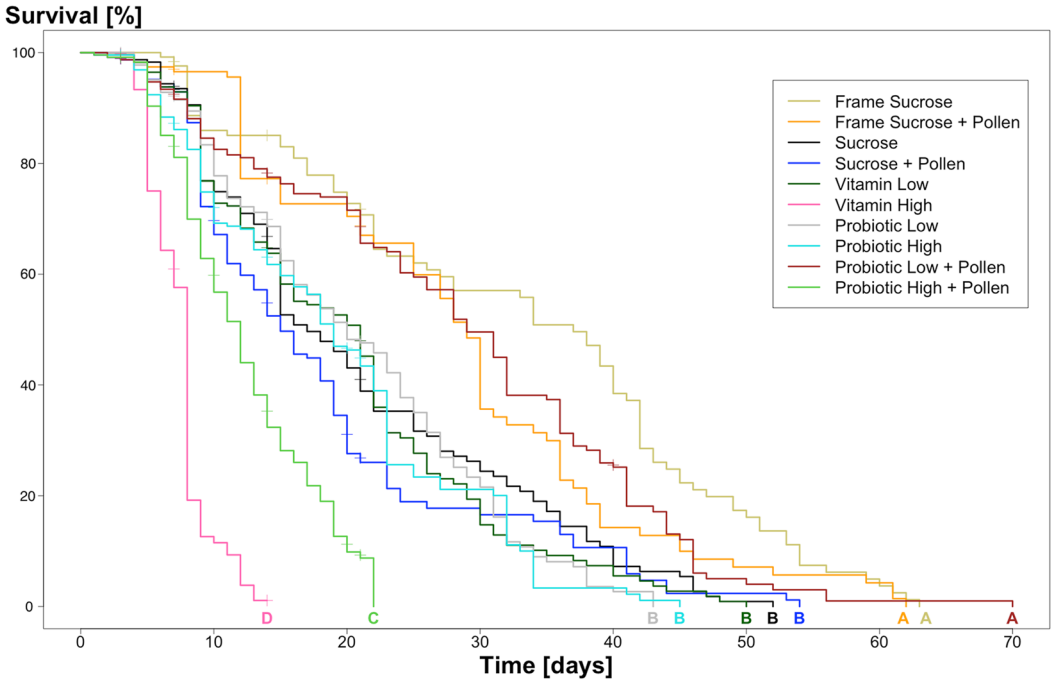
**Figure 4.** Kaplan–Meier survival curves of two groups of experimental summer workers, one of which was treated with tetracycline at dosages of 500  $\mu\text{g}/\text{mL}$  (Tetracycline-500) supplied in sucrose solution (50%, w/v) and a control supplied with sucrose solution only (50%, w/v).  $N=180$  workers per treatment group (6 cages/group with 30 bees each).  $N=360$  total workers. Significant differences between the survival of the experimental workers based on chi-squared testing ( $\rho=0$ ) are indicated by compact letter display ( $p < 0.05$ ).

Holm adjusted  $p$  values, all  $p_s < 0.05$ ) showed “Frame Sucrose + Pollen” group as consuming consistently the most across all other treatments (Fig. 2, letter C), while all other groups consumed statistical similar amounts (Fig. 2, letters A, B, and AB). A multiple regression model ran with indicator variables, excluding “Frame,” was performed and the model summary reveals that high levels of b-vitamins negatively influenced sucrose consumption (lmer, coefficient  $-0.0042$ ,  $p = 0.05$ ) as well as low levels of probiotics

(lmer, coefficient  $-0.0039$ ,  $p < 0.05$ ), and finally that there were no significant interactions.

### 3.4. Tetracycline assessment

The winter bees from the first hoarding cage trial assessment of tetracycline had a lifespan of 1 to 41 days ( $N=3$  treatment groups,  $N=3$  replicates/treatment,  $N=30$  workers/treatment,  $N=90$  total workers/group,  $N=270$  total workers). No



**Figure 5.** Kaplan–Meier survival curves of experimental workers, *Apis mellifera*, from 10 treatment groups, of which eight received a 72-h tetracycline treatment: Sucrose, Sucrose+Pollen, Probiotic Low, Probiotic High, Probiotic Low + Pollen, Probiotic High + Pollen, Vitamin Low, and Vitamin High ( $N=232$  workers per group). The two groups: Frame Sucrose and Frame Sucrose+Pollen, remained 72 h on their frame (no antibiotic treatment,  $N=116$  workers per group). Significant differences between the survival of the experimental workers are indicated by compact letter display based on log rank tests and Bonferroni  $p$  adjusted values ( $ps < 0.05$ ).

significant differences were found between the non-antibiotic-treated control “Sucrose” and the two tetracycline dosages, Tetracycline-400 ( $400 \mu\text{g/mL}$ ) and Tetracycline-700 ( $700 \mu\text{g/mL}$ ) (Kaplan–Meier, log rank test, all  $ps > 0.05$ , letter A, Fig. 3).

The summer bees from the second hoarding trial assessment of tetracycline had a lifespan of 1 to 55 days ( $N=2$  treatment groups,  $N=6$  replicates/treatment,  $N=30$  workers/treatment,  $N=180$  total workers/group,  $N=360$  total workers). No significant difference was found between the non-antibiotic-treated control “Sucrose” and the tetracycline dosage, Tetracycline-500 ( $500 \mu\text{g/mL}$ ) (Kaplan–Meier, log rank test, all  $ps > 0.05$ , letter A, Fig. 4).

### 3.5. Longevity

The experimental workers from the ten treatment groups lived between 1 and 70 days ( $N=1856$  workers in tetracycline-treated groups and  $N=232$  workers in “Frame” treatment groups, total  $N=2088$ , Fig. 5). Log rank testing resulted in significant differences in survival probability through time between groups (Table III,  $p$  value  $< 0.001$ ). Best survival was observed in the three treatment groups “Frame Sucrose” and “Frame Sucrose + Pollen,” and “Probiotic Low + Pollen,” which had statistically similar outcomes (Kaplan–Meier, log rank test, all  $ps > 0.05$ , letter A, Fig. 5). No significant differences were found between the negative and positive controls

**Table III** Observed, expected, and chi-square values ( $\chi^2$ ) derived from the log rank tests from the survival analysis, with the sum of  $\chi^2$  highlighted in bold. The resulting  $p$  value was  $p < 0.001$

| Treatment               | Observed (O) | Expected (E) | $\chi^2: (O - E)^2/E$ |
|-------------------------|--------------|--------------|-----------------------|
| Frame Sucrose           | 88           | 181.5        | 48.133                |
| Frame Sucrose + Pollen  | 80           | 130.4        | 19.449                |
| Sucrose                 | 160          | 170.6        | 0.66                  |
| Sucrose + Pollen        | 155          | 135.4        | 2.824                 |
| Vitamin Low             | 153          | 157.5        | 0.128                 |
| Vitamin High            | 198          | 41.6         | 587.867               |
| Probiotic Low           | 151          | 159          | 0.403                 |
| Probiotic High          | 144          | 137.4        | 0.32                  |
| Probiotic Low + Pollen  | 145          | 242.3        | 39.091                |
| Probiotic High + Pollen | 171          | 89.3         | 74.632                |
| Sum ( $\chi^2$ )        |              |              | <b>922</b>            |

“Sucrose” and “Sucrose + Pollen,” as well as the treatment groups “Probiotic High,” “Probiotic Low,” and “Vitamin Low” (Kaplan–Meier, log rank test, all  $ps > 0.05$ , letter B, Fig. 5). Compared to these groups, “Probiotic High + Pollen” showed a significantly higher mortality (Kaplan–Meier, log rank test, all  $ps < 0.05$ , letter C, Fig. 5). Workers that received “Vitamin High” as a treatment showed the highest mortality, significantly higher than all other treatment groups (Kaplan–Meier, log rank test, all  $ps > 0.05$ , letter D, Fig. 5). The survival data showed that the treatment outcomes were dosage-dependent. The two vitamin concentrations either had no effect on longevity (“Vitamin Low”) or negatively affected longevity of the workers (“Vitamin High”) when compared to the control groups: “Sucrose” and “Sucrose + Pollen” (Kaplan–Meier, log rank test, all  $ps > 0.05$ , letters “D” and “B,” Fig. 5). In the absence of pollen, the probiotic treatments had statistically similar survival outcomes as the control groups; however, when the probiotics were supplied together with pollen, the effects did significantly change (Kaplan–Meier, log rank test, all  $ps > 0.05$ , letters “C” and “A,” Fig. 5). The observed effect was positive for the combination “Probiotic Low + Pollen” and negative for “Probiotic High + Pollen.”

#### 4. DISCUSSION

The data obtained here provide no evidence for a beneficial effect of the applied dosages of b-vitamin or probiotic supplementation on longevity and body weight of antibiotic-treated honey bee workers. The data further demonstrate that supplementing sucrose solution fortified with b-vitamins or probiotics (*Lactobacillus* spp.) can significantly influence workers’ longevity, and above all, that *ad libitum* access to pollen as well as natural post-emergent gut flora inoculation (i.e., Frame) are key to increasing longevity, underscored by the two “Frame” groups as well as “Probiotic Low + Pollen.” Interestingly, this favorable outcome was distinctly dosage-dependent, drawing a striking contrast between “Probiotic High + Pollen” vs. “Probiotic Low + Pollen,” and similar consequences were observed in the two vitamin dosages “Vitamin High” vs. “Vitamin Low,” where in both instances, the high dosages proved to be detrimental. Finally, the data provide supporting evidence for the importance of pollen as well as an intact gut microbiota on worker body weights, where the “Frame Sucrose + Pollen” weighed the most of any group.

In *A. mellifera* studies, both fresh and dry weights have been recommended as measurable health parameters (EFSA, 2016), and here, dry weight was used for statistical analyses because it excludes water weight values and therefore, yields closer-to-true biomass data. The body weight results of this study suggest a significant positive effect from *ad libitum* access to pollen, while the Frame Sucrose + Pollen bees with an intact gut-microbiota weighed the most. Considering that pollen is both a substantial part of honey bee diets and has a high nutritive value of both macro- and micronutrients necessary for development, coupled with the gut microbiota's role in aiding digestion (Kwong & Moran, 2016), the likelihood of such an outcome is expected and coheres with previously published literature (Haydak, 1970; Roulston & Cane, 2000). Interestingly, pollen had differing inter-treatment effects on worker weights, where in most cases, it was beneficial. However, in one particular case, the experimental workers that were part of the treatment "Probiotic High + Pollen" scored statistically worse than their counterpart "Probiotic Low + Pollen," and ultimately finished in the lowest weight category. The linear model results also suggest a highly significant antagonistic interaction between pollen and probiotics in high dosages. It is important to outline that these workers were continually fed artificially elevated numbers of *Lactobacillus* bacteria while retaining *ad libitum* access to pollen, a known energy source for *Lactobacillus* bacteria (Vásquez & Olofsson, 2009), and it is possible this created an ideal scenario for *Lactobacillus* overgrowth. Such instances of gut dysbiosis have been shown to be harmful in other taxa, such as impeding digestion in mice (Hayashi et al., 2017), and has also been correlated with increased mortality in honey bees (Maes et al., 2016). Additionally, *Lactobacillus* bacteria produce lactic acid as a byproduct of carbohydrate digestion, creating a low pH environment favorable to *Lactobacillus* bacteria, giving them an additional advantage for out competing other flora (Sanhueza et al., 2015), but harmful for the bees.

The results of the body weight data are complimented by the sucrose-consumption data,

where the "Frame Sucrose + Pollen" group both consumed and weighed the most. Honey bees strive to maintain a stable protein to carbohydrate (P/C) ratio, where an unbalanced P/C ratio, such as too high levels of protein, can have detrimental consequences (Altaye et al., 2010). Although pollen was not a significant predictor for sucrose consumption, pollen consumption data would have allowed the present study to properly perform a correlation analysis in order to confirm if a stable P/C ratio was indeed present here.

The longevity results demonstrate that the cage conditions seem to drastically alter the average lifespan of winter bees. Under natural conditions, winter bees normally live 150 days or longer (Fukuda & Sekiguchi, 1996), yet here, the oldest workers reached 70 days, accentuating an inherent flaw with the cage design for studying winter bees. Collectively, this draws attention to the difficulty of translating effects observed in laboratory trials to predicted outcomes in natural hive conditions, and published efforts to make hoarding cage improvements have been done (Huang et al., 2014). Despite here applied measures such as randomization of workers to control for genetic variation between colonies, as well as the imitation of in-hive conditions (i.e., temperature, light, etc.), many aspects still diverge from a natural hive scenario.

In line with this, the workers that were exposed to the closest to real-life scenario, the "Frame" groups, had the best survival outcomes, concurrent with the "Probiotic Low + Pollen" group. The underlying commonalities between the longest-lived workers in this study were microbiota (i.e., "Frame" and probiotic groups) and, most importantly, pollen. Nutrition-based interactions with a host's microbiota are well established in animals (Bäckhed et al., 2005; Douglas, 2018), and in general, it is recognized that an insect's microbiota facilitates many beneficial interactions, such as digestion facilitation (Brune, 2014), vitamin production (Salem et al., 2014), and detoxification (Kešnerová et al., 2017). Vásquez et al. (2012) found that nine different *Apis* species have estimated total ranges of  $10^6$ – $10^7$  *Lactobacillus* bacteria in their honey

crop, with similar spectrums of lactobacilli found in honey, pollen, and bee bread. The “Probiotic Low + Pollen” group received concentrations in these ranges in their diet following a tetracycline treatment, and it is arguable that this was the proper dosage for restoring vital microbial-gut symbiotic functions. In contrast to the latter group, all other workers that underwent the antibiotic treatment showed higher mortality, even though no negative effects on survival were observed in pre-trials of this treatment method. Additionally, it is noteworthy to state that the workers in the “Probiotic High + Pollen” group had a severe decrease in lifespan, likely due to the previous mentioned overabundance of lactobacilli from being fed excessive high doses of bacteria. Finally, microbial endosymbionts in insects can be highly host-specific (Xu et al., 2020), and Lactibiane Voyage contains probiotic species targeted for human digestion. Alternative solutions with probiotics containing bee-specific bacterial strains would be an interesting area of investigation for honey bee health.

Although tetracycline should not be harmful to *A. mellifera* because it inhibits protein synthesis in prokaryotic organisms (Chopra & Roberts, 2001) in addition to our longevity pre-trial data results being analogous with the aforementioned statement, such instances can nonetheless not be ruled out. Long-term consequences associated to antibiotic exposure in bees has been documented in other studies, notably increased mortality (Marceau et al., 2021; Raymann et al., 2017), disruption in protein digestion (du Rand et al., 2020; Retschnig et al., 2021), and negative influence on sucrose consumption (Retschnig et al., 2021). The pairwise comparison results from the sucrose consumption data (Fig. 2) demonstrate that no differences were found between the “Frame Sucrose” and the micronutrient or probiotic supplemented bees, but the lack of a non-tetracycline without “frame” treatment limits formal statistical conclusions if there were true negative effects on sucrose consumption or body weight from antibiotic exposure.

The “Vitamin High” group was the worst performing treatment, with the lowest likelihood of survival. Shortly after starting the experiment,

there was an appearance of trembling, falling, and behavioral agitation in their cages compared to their counterpart “Vitamin Low” and all other treatment groups, hinting at possible signs of toxicity, as seen in honey bees (Suchail et al., 2004; Ludicke & Nieh, 2020) (Fig. 6). This was strictly an anecdotal observation, and it would have been beneficial to this study to have had followed formal laboratory protocols to measure such abnormal activity. Interestingly, *A. mellifera* lifespans have been correlated with energy expenditure (Neukirch, 1982), and it is possible the “Vitamin High” workers over exhausted themselves resulting in premature death. Intriguingly, it may not just be the high dosages of b-vitamins themselves that were pernicious, but a combined habitual long-term exposure to them over extended periods of time which ended up being harmful. Such instances have been seen in other taxa, such as ataxia, muscle weakness and loss of balance in beagles (Phillips et al., 1978), osteo-related complications in humans (Meyer et al., 2019) as well as many other harmful ailments in humans due to heightened long-term consumption of b-vitamins (Titcomb & Tanumihardjo, 2019). Large taxonomic differences between mammals and insects exist; however, inferring ideas from mammalian studies offer important reasons for speculation and thus, emphasizes the need for future studies around the idea of b-vitamin dosages in *A. mellifera* studies to avoid toxicity issues. Lastly, honey bees, when kept in cages, do not defecate (Köhler et al., 2013), blocking the pathway of expelling amassed waste in their rectums, a studied trigger of early death in honey bees (Altaye et al., 2010). This aspect may have led to accumulated high dosages of b-vitamins in the experimental workers of the present study and has possibly resulted in the observed harmful effects and contributed to the decreased lifespan.

The present study provides no direct evidence of a beneficial effect solely from the concentrations of the supplements used on the investigated health parameters in antibiotic-treated winter bees. The most beneficial effects were observed in the workers subjected to the closest real-life scenario (i.e., access to pollen and natural post-emergent gut flora inoculation). Conversely,



**Figure 6.** Video of experimental honey bee workers, *Apis mellifera*, in hoarding cages. (A) B-vitamin Low treatment (300 mg/L), (B) Sucrose (control), and (C) B-Vitamin High treatment (9 g/L). The treatment workers (C) display clinical symptoms of B-vitamin poisoning in honey bees (hyperactivity; URL: <https://youtu.be/450-eQjvBUE>).

strong detrimental effects were observed for high amounts of b-vitamins and probiotics, highlighting caution before considering any field application of micronutrients at our studied concentrations. Combined efforts of improving hoarding cage designs in parallel with range-finding tests to determine micronutrient dosage safety are of utmost importance in future studies.

### SUPPLEMENTARY INFORMATION

The online version contains supplementary material available at <https://doi.org/10.1007/s13592-022-00927-4>.

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### AUTHOR CONTRIBUTION

AB, GR, and PN designed and AB, VR, and JP conducted the experiment; AB and GR analyzed the data; AB, GR, and PN wrote the manuscript; and VR, JP, and VP revised and approved the final manuscript.

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### DATA AVAILABILITY

The raw data of the study are available at the Dryad repository: <https://doi.org/10.5061/dryad.b5mkkwhdq>.

### CODE AVAILABILITY

All statistical code used for the present study is found in the supplementary material (see Supplementary Material, R script).

### DECLARATIONS

**Ethics approval** No approval of research ethics committees was required to accomplish the goals of this study because experimental work was conducted with an unregulated invertebrate species.

**Consent to participate** Not applicable.

**Consent for publication** All authors have read and approved the manuscript.

**Conflict of interest** The authors declare no competing interests.

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