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Assessment of mutualism between *Bombus terrestris* and its microbiota by use of microcolonies

Ivan MEEUS¹, Veerle MOMMAERTS¹, Annelies BILLIET¹, Hadi MOSALLANEJAD¹,
Tom Van de WIELE², Felix WÄCKERS³, Guy SMAGGHE¹

¹Department of Crop Protection, Faculty of Bioscience Engineering, Ghent University, Coupure Links 653, 9000 Ghent, Belgium

²Laboratory of Microbial Ecology and Technology, Faculty of Bioscience Engineering, Ghent University, Ghent, Belgium

³Biobest NV, Westerlo, Belgium

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Abstract – Social pollinators, like honeybees and bumblebees, have specific microbiota. The specific association between the insect and its gut microbiota could lead to a functional dependence. In this project, we investigated whether an alteration in microbiota could influence the fitness of the generalist pollinator *Bombus terrestris*. We used bumblebee workers in microcolonies to assess effects on colony development and reproduction. Treatment with 200 ppm streptomycin and tetracycline resulted in a disruption of gut microbiota, and typically there was 100 % worker mortality. In contrast, treatment with streptomycin alone showed an improved reproduction and this concurred with a different gut microbiota pattern. The results are discussed in relation to effects of the gut microbiota on bumblebee fitness and potential beneficial effects by *Lactobacillus* sp. These data may open new avenues in the mass-rearing of bumblebees used for the biological pollination in agriculture.

host–microbe interactions / social pollinators / *Bombus terrestris* / streptomycin / *Lactobacillus* / probiotica

1. INTRODUCTION

Insects are arguably the most successful group of animals on the planet, occupying a diverse range of ecological niches. This ecological success is often facilitated by bacterial symbionts (Brownlie and Johnson, 2009). These acquired microbial genomes, among others, can synthesize required nutrients and cofactors for the insect host, enable the host to utilize

unusual or low-nutrient diets, give protection against parasitism and/or influence patterns of host plant use (Dillon et al., 2005; Moran et al., 2008; Riegler and O'Neill, 2007). Within the *Apidae*, a family of bees with an important ecological value in maintaining plant diversity and providing pollination services (Ghazoul, 2005; Steffan-Dewenter et al., 2005), especially honeybees and bumblebees are well-studied. This is due to their economic value for the cultivation of pollinator-dependent crops (Aizen et al., 2008; Velthuis and van Doorn, 2006). The role of beneficial host–microbe interactions of social insects is currently under investigation. The honeybee and bumblebee gut microbiota has recently been identified, revealing a highly specific, yet overlapping community composition with low species diversity (Koch and Schmid-Hempel,

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Corresponding author: I. Meeus,
ivan.meeus@ugent.be
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2011a; Martinson et al., 2011; Koch et al., 2013). In other insects, the microbiota may be more variable within a single species. Indeed, the gut microbiota of common fruit flies from different locations showed variable species richness (Corby-Harris et al., 2007; Chandler et al., 2011), and diet plays a major role in shaping the gut microbiota of larvae of the gypsy moth (Broderick et al., 2004). The specific relation between the gut microbiota and social pollinators creates the opportunity to evolve a functional dependence. Progress has been made in elucidating the functionality of this specific microbiota in social pollinators. A metagenomic analysis revealed genes involved in biofilm formation and pectin-degrading enzymes (Engel et al., 2012). The gut microbiota is also involved in immunity; good examples are the protection against the gut parasite *Crithidia bombi* in bumblebees (Koch and Schmid-Hempel, 2011b) and American Foulbrood in honeybees (Forsgren et al., 2010). In summary, beneficial interactions between bacteria and social pollinators are to be expected.

In this project, we investigated this essential host–microbe interaction to see whether alterations of the microbiota in the gut of bumblebee workers can influence their fitness. We used *Bombus terrestris*, a eusocial generalist pollinator with an annual life cycle which is one of the most numerous bumblebee species in Europe and also commercially mass-reared for biological pollination in greenhouses particularly in tomatoes (Velthuis and van Doorn, 2006). This species offers the unique opportunity to use microcolonies, a standardized setup to study colony development and reproduction following exposure to different treatments (Mommaerts et al., 2006). Although microcolonies have already been used before in insecticide testing, we applied them for the first time to test whether the microbiota patterns in the gut of these workers are representative for a queen-right colony. With these microcolonies, we then determined whether antibiotic treatments like streptomycin and tetracycline can induce changes in the microbiota of bumblebees compared to untreated microcolonies. Subsequently, we scored the fitness of these microcolonies to evaluate whether changes in microbiota affect colony development and reproduction in order to identify certain beneficial bacterial species.

2. MATERIALS AND METHODS

2.1. Insects

All bumblebees were obtained from a continuous mass rearing program (Biobest, Westerlo, Belgium) and fed on commercial sugar water, containing methyl and propyl hydroxybenzoate at 0.6 % (BIOGLUC®, Biobest) and honeybee-collected pollen (Soc. Coop. Apihurdes, Pinofranquedo-C'aceres, Spain) as energy and protein source, respectively. The insects were kept under standardized laboratory conditions with 28–30 °C, 60–65 % relative humidity, and continuous darkness (Mommaerts et al., 2006).

2.2. Microcolonies and measuring fitness of microcolonies

We used microcolonies to quantify fitness effects of different antibiotic treatments on bumblebees. These microcolonies are created by placing newborn workers (exact numbers of bumblebees are given in each specific experiment) in an artificial nest box (15×15×10 cm). After 1 week one worker becomes dominant and starts laying unfertilized eggs that develop into drones while the other workers take care of the brood and forage for food. These microcolonies follow a well-defined development pattern with quantifiable parameters (i.e., time point of egg laying, larval development time, pupation time, reproductive output, and larval mortality). Microcolonies contain unfertilized workers that only produce male offspring. Hence the reproduction output can be determined by counting the numbers of drones and by weighing (Adventurer Pro AV413C) their mass. The microcolonies were kept at the standardized rearing conditions as reported above for the rearing.

Normal distribution of data was confirmed by Kolmogorov–Smirnov test ($P=0.05$) and the mean±SD were analyzed by an independent-sample t test ($P=0.05$).

2.3. Microbiota of workers in microcolonies compared to queen-right colonies

Seven newborn workers were selected from one queen-right colony and placed in one microcolony box. This setup was performed for six queen-right colonies as biological replicates ($n=6$). From each microcolony, we sampled one 4-day-old and one 8-day-old worker to

determine the gut microbiota as described below. From the queen-right colony, the newborn workers were labeled and placed back in the colony. Then, three 0-day-old, three 4-day-old, and three 8-day-old workers were sampled per colony and we characterized the gut microbiota as described below.

2.4. Fitness and gut microbiota of workers in microcolonies treated with 200 ppm streptomycin and tetracycline

Each microcolony started with seven newborn workers coming from one queen-right colony. Per queen-right colony ($n=10$), we started a control group (C; $n=10$), and a continuous antibiotic treatment with streptomycin and tetracycline (S+T; $n=10$). The two antibiotics streptomycin and tetracycline were added at 200 ppm in the diet, via treated sugar water and treated pollen as described in Mommaerts et al. (2006).

Fitness parameters of each microcolony were recorded as described above. For six microcolonies per treatment two bumblebees were sampled (at days 4 and 8) to determine the microbiota.

2.5. Food uptake by workers after 200 ppm streptomycin and tetracycline treatment

To investigate how the feeding of bumblebee workers with streptomycin- and tetracycline-treated diet (both at 200 ppm) affected the amount of food taken up by microcolonies, we followed food uptake in 20 microcolonies each originating from 10 randomly selected newborn workers. Firstly, all 20 received food ad libitum for 4 days. Then the experiment started (day 0), ten microcolonies were provided with treated sugar water and pollen (treatment S+T, as described above). As a negative control ten microcolonies were fed untreated sugar water and pollen. The mass of the pollen and sugar water was determined by weighing them on a digital microbalance (Adventurer Pro AV413C) before administering and reweighing after 1 day (consumption on day 1) and after 3 days (consumption of days 1–3). Thereafter, fresh pollen and sugar water were provided (control versus treatment), again mass was determined before administering and 3 days later (consumption of days 4–6). Normal distribution of food consumption data was confirmed by Kolmogorov–Smirnov test ($P=0.05$) and the means $\pm$ SD were analyzed by an independent-sample t test ($P=0.05$).

2.6. Fitness and gut microbiota of workers in microcolonies treated with 200 ppm streptomycin

Twenty microcolonies were started with six newborn workers that were randomly selected from different queen-right colonies. Two different treatment groups were created each consisting of ten microcolonies: control microcolonies (C) were fed normal untreated sugar, while in treated microcolonies the sugar water was supplemented with 200 ppm streptomycin (S). Fitness parameters of each microcolony were recorded as described above. To determine the gut microbiota we sampled one 8-day-old worker from each of the 20 microcolonies.

2.7. Characterization of the gut microbiota

2.7.1. DNA extraction

The mid and hindgut regions of the worker bumblebee were separated from the crop and stored individually in 200 μ l of acetone for later analysis. Stored samples were washed three times with sterile phosphate-buffered saline (PBS, pH 7.4). For this purpose the gut was opened by gentle crushing in a microtube using a Teflon pestle in 200 μ L of PBS and then spun down at 10,600 $\times g$. The pellets were incubated at 37 °C for 2 h in a 170- μ L lysozyme solution (100 mg/ml) followed by treatment with 25 μ l of Proteinase K (Sigma-Aldrich) for 1 h at 56 °C. In order not to preferentially extract gram negative bacteria, the samples were transferred to 300 μ l of lysis buffer from the EZNA® Insect DNA Kit (Omega Bio-Tek) supplemented with 0.3 g of zirconia beads (diameter 0.1 mm; BioSpec Products) and further homogenized by bead beating in the Fastprep TM FP 120 (Thermo Scientific). Further extraction was performed according to manufacturer's instructions.

2.7.2. Fingerprinting the microbial community

We generated a 16S rDNA gene molecular fingerprint of the microbiota of the bumblebee gut in order to analyze its structure and diversity. The bacterial 16S rDNA was amplified without co-amplification of predominant eukaryotic DNA by nested PCR with the

primers Eub8F and 984R for external PCR and 338F-GC and 518R for internal PCR (Bakke et al., 2011). The reaction mixture contained 2 mM MgCl_2 , 0.3 mM dNTP, 0.3 μM primers, recombinant Taq DNA polymerase (Invitrogen), and 5 μL of DNA extract for the external PCR and 1 μL (1/50 diluted external PCR product) for the internal PCR. A total of 25 μL of internal PCR reaction mix was placed in the Sensoquest Labcycler for 2 min at 94 °C followed by 21 amplification cycles (30 s at 94 °C, 30 s at 50 °C, and 60 s at 72 °C) and then by 3 min at 72 °C. For the external PCR, the same procedure was followed, except the annealing temperature was 53 °C, 25 amplification cycles were used and the final elongation was extended from 3 to 20 min to complete the polymerization.

The 16S rDNA gene molecular fingerprint was generated by denaturing gradient gel electrophoresis (DGGE). Ten microliters of the PCR products were separated on an 8 % (w/v; 40 % acrylamide, 2 % bisacrylamide) gel with a denaturing gradient of 45 to 60 %; 100 % denaturation refers to 7 M urea and 40 % deionized formamide. Electrophoretic separation was performed on the INGENYphorU (Ingeny) for 16 h at 120 V in 1× TAE buffer (20 mM Tris, 10 mM acetate, 0.5 mM EDTA pH 7.4) at 60 °C. The gels were stained using SYBR Green (1:10,000 dilution, FMC BioProducts) in 1× TAE for 20 min and visualized by UV transillumination (Vilbert Loumat).

2.7.3. Diversity analysis

Statistical comparison of the DGGE patterns was done with Bionumerics software (Applied Maths, Kortrijk, Belgium). Similarity matrices were based on the Pearson correlation coefficient using data from matched bands. Dendrograms were calculated using the clustering algorithm of Ward (Van de Wiele et al., 2004). Differences in similarity were calculated by analysis of similarities (ANOSIM) (Clarke and Green, 1988; Clarke, 1993) using Primer version 6.1.10 software. It calculates a global R statistic which lies between -1 and +1, with high values indicating a large degree of discrimination among groups.

The structural diversity of the microbiota is characterized by the Shannon index of general diversity H (Vervaeren et al., 2006). The equation for the Shannon index is: $H = -\sum (n_i/N) \log(n_i/N)$, where n_i is the height

of the peak and N the sum of all peak heights of the densitometric curve (Vervaeren et al., 2006).

Microbiota diversity analysis was based on the method of Lorenz as described (Mertens et al., 2005; Wittebolle et al., 2009). In short, two parameters were derived from the bacterial fingerprint. Firstly, species richness (R), which is the number of bands. Secondly, the community organization (Co), for which a low number (for example 25 %) represents a community with high evenness or with no specific dominant species (Marzorati et al., 2008).

For all data, normal distribution was confirmed by Kolmogorov–Smirnov test ($P=0.05$) and then the means $\pm$ SD were analyzed by an independent-sample t test ($P=0.05$) or one-way ANOVA and separated by a post hoc Tukey–Kramer test ($\alpha=0.05$) using SPSS v. 16.0.

2.7.4. Identification of bacteria

Bacterial species were identified by cloning the external PCR fragment of one control bumblebee and one streptomycin-treated bumblebee from a microcolony into a pJET1.2/blunt vector with the CloneJET PCR Cloning Kit (Thermo Fisher Scientific) following manufacturer's instructions. Afterwards, plasmids were transformed in competent *Escherichia coli* XL-1 Blue Cells by heat shock and then plated out on a carbenicillin-containing LB agar plate. After 16 h incubation, only three and two colonies were formed, respectively. From each clone a plasmid was purified using the Plasmid mini prep kit (Omega Bio-Tek) and sent for sequencing to LGC Genomics (Berlin, Germany). The place of the bacterial plasmids on the DGGE profile was determined after nested PCR with external vector-specific primers and the internal primers as described above.

3. RESULTS

3.1. Microbiota of workers in microcolonies compared to queen-right colonies

The development of the microbiota was followed in untreated queen-right colonies ($n=6$) and from each colony we sampled nine workers [three newborn workers (callow workers) and three

workers of 4 and 8 days old, respectively]. Newborn workers showed hardly any microbiota in their gut with a species richness R of 0.17 ± 0.41 . By day 4, the gut microbiota had developed and was significantly different from day 0 (R , $P < 0.05$, ANOVA). Over the subsequent 4 days, the community richness $R = 10.14 \pm 3.62$ ($P = 0.53$), the Shannon index of general diversity $H = 0.87 \pm 0.18$ ($P = 0.43$), and the community organization $Co = 35.89 \pm 4.77$ ($P = 0.73$) remained stable (day 4 compared to day 8; ANOVA; Figure 1a). Calculating the same parameters, we could not detect any difference between bumblebees from microcolonies ($n = 6$) (days 4 and 8) and those from queen-right colonies ($n = 6$) at days 4 and 8 (ANOVA, R : $P = 0.94$; H : $P = 0.80$; and Co : $P = 0.49$; Figure 1a). However, looking at the similarity of the DGGE patterns of queen-right colonies (74.8 ± 12.8) and microcolonies (77.5 ± 14.0) a higher similarity was found opposed to the similarity between queen-right colonies and microcolonies (62.9 ± 9.4 ; Figure 1b). ANOSIM, comparing each queen-right colony with its corresponding microcolony, revealed some separation ($R = 0.33 \pm 0.26$), but none were significantly different. However, some were close to the $P = 0.05$ (i.e., $P = 0.07$; $P = 0.14$; $P = 0.10$; $P = 0.33$; $P = 0.70$; $P = 0.21$). Although most bands found within microcolonies corresponded with those found in queen-right colonies, we speculate that the early separation of callow workers from the nest has a minor effect on the development of the microbiota.

3.2. Fitness and gut microbiota of workers in microcolonies treated with 200 ppm streptomycin and tetracycline

We assessed the effect of a continuous antibiotic treatment with streptomycin and tetracycline (S+T) on the microbial development compared to no exposure to antibiotics (C). The microbiota was assessed at days 4 and 8 of six of the ten microcolonies (per treatment). While the bumblebees of the untreated microcolonies developed a microbiota as described above, no bands were detected in the DGGE patterns of the treated bumblebees. This indicates that after 4 and 8 days of treatment no bacteria had developed.

In parallel to the gut microbiota analysis, the fitness of these microcolonies was measured. The mixture of antibiotics resulted in 100 % worker mortality after 4 weeks. In contrast, no mortality was scored after 50 days in the untreated control groups (C).

3.3. Food uptake by workers after 200 ppm streptomycin and tetracycline treatment

As shown in Figure 2, the food uptake of microcolonies having been fed an antibiotic-containing diet for 1 day was not significantly different as compared to the control group (for sugar water: independent sample t test, $P = 0.23$; for pollen: independent sample t test, $P = 0.36$). This demonstrates that the antibiotic-treated food did not have a direct antifeedant effect during the first day of treatment.

During the experiment, the colonies treated with streptomycin and tetracycline showed a decrease in sugar water and pollen consumption when comparing the mean consumption from day 1 to day 3 and from day 4 to day 6 (independent sample t test; $P < 0.05$ for sugar water and $P < 0.05$ for pollen; Figure 2). In contrast, the sugar water consumption in untreated colonies increased as the workers of the colony started to make sugar pots.

3.4. Fitness and gut microbiota of workers in microcolonies treated with 200 ppm streptomycin

We recorded a shift in the microbiota of bumblebees receiving 200 ppm streptomycin (S; $n = 10$; day 8) compared to control microcolonies (C; $n = 10$; day 8). First, the general diversity of the microbiota dropped sharply as illustrated in Figure 3b by the Shannon index H (independent t test with unequal variance $P < 0.001$) and species richness R (independent t test, $P < 0.001$). Second, the bacterial identity was also different with control (C) bumblebees clustering separately from streptomycin-treated ones (S; Figure 3a). The bands highlighted in red (Figure 3a) are unique to the streptomycin-treated colonies. Indeed their relative position in the DGGE gel is different from

Figure 1. **a** The community richness R , the Shannon index of general diversity H , and the functional organization Fo of bumblebee workers from queen-right colonies versus microcolonies. **b** Similarity of queen-right colonies and microcolonies and between queen-right colonies and microcolonies, based on the Pearson correlation coefficient using data from matched bands

the two closest bands present in the untreated control samples (see supplementary information Table S1). The red colon punctuations in Figure 3a also represent unique bands in the streptomycin treatments, but these have a very weak intensity that was only visible upon contrast and brightness adjustments.

The two red bands unique for the streptomycin-treated samples were identified as the same bacterial species, namely *Lactobacillus* sp. (deposited at GenBank as KC477412) as had been previously isolated from the honeybee (GenBank HM534867). The bands mainly present in untreated bumblebees and here represented in blue are a *Snodgrassella* sp. (deposited at GenBank as KC477411) and a *Bifidobacterium* sp. (deposited at GenBank as

KC477410), both of which had previously been identified in wild bumblebees (Koch and Schmid-Hempel, 2011a,b).

When evaluating the fitness effects of the 200 ppm streptomycin treatment (S; $n=10$), we observed no loss of survival of treated workers and also a normal development of the microcolony: the first egg was laid at 8 ± 1 days, the first pupa was formed at day 21 ± 1 and the first drone emerged at day 33 ± 1 (independent sample t test; first egg: $P=0.14$, first pupa: $P=0.50$, first drone $P=1.0$). However, the numbers of drones that emerged after 50 days were significantly increased by about 20 % from 32.8 ± 5.9 in the untreated colonies to 39.2 ± 4.3 in streptomycin-treated colonies (S; independent sample t test, $P<0.05$). Interestingly,

Figure 2. Mean individual sugar water (μl) and pollen (mg) consumption of ten bumblebees per colony ($\pm\text{SD}$ of ten colonies). *Equal signs* addition of streptomycin and tetracycline to sugar water and pollen does not reduce consumption at day 1 ($n=10$). *Asterisks* a drop in consumption ($P<0.05$) is recorded when comparing the average consumption in days 1, 2, and 3 ($n=10$) with 4, 5, and 6 ($n=10$)

there was also an increase of more than 15 % in the individual drone mass with 345 ± 57 mg in the streptomycin-treated colonies compared to 293 ± 59 mg in the untreated colonies (independent sample t test, $P<0.05$; Figure 4).

4. DISCUSSION

In order to create an altered microbiota in microcolonies we used an antibiotic treatment. First we used a continuous treatment with two broad range antibiotics, namely streptomycin and tetracycline both at 200 ppm. This resulted in a total eradication of gut bacteria and a dramatic negative impact on bumblebee fitness, resulting in 100 % mortality (at week 4) compared to no mortality in bumblebees with a microbiota. One reason that we observed mortality in bumblebee

workers treated with these antibiotics could be that the bumblebees died due to starvation because the treated food may have posed an antifeedant effect. We measured no difference in food uptake between treated and control colonies in the first day after administering the antibiotics, which allows the conclusion that antibiotic-supplemented food had no direct antifeedant effect on the bumblebees (Figure 2). But in continuation of the experiment, the bumblebees from the antibiotics-treated colonies showed a loss of appetite between days 1–4 and days 4–6 as expressed in a reduced uptake of both pollen and sugar water (Figure 2). Two possibilities remain to explain the loss of appetite followed by the death of the workers. The antibiotics had a direct toxic effect. Although similar dosage of antibiotics is regularly used to preserve food for insect mainte-

Figure 3. **a** The dendrogram shows that major bacterial species mainly disappear after streptomycin treatment. Bands highlighted in black are unique in streptomycin samples. Colon punctuations represent unique low-intensity DNA bands (cropped image below shows the presence of the low-intensity DNA bands after adjusting contrast and brightness). The grey box shows overlapping expression between two streptomycin-treated samples with the control. The bands in the grey boxes of streptomycin-treated samples show lower expression compared to the band representing *Lactobacillus* sp. (GenBank KC477412). **b** Significant drop in species mean richness $R \pm SD$ ($n=10$; $P<0.001$) and the mean Shannon index $H \pm SD$ ($n=10$; $P<0.001$) after streptomycin treatment

nance under laboratory conditions or to cure insects from endosymbionts (Breeuwer and Werren, 1990; Wilkinson, 1998), it is also known that tetracycline can have host toxicity, mainly by inhibiting the mitochondrial protein synthesis (McKee et al., 2006). Therefore previous reports have assigned mortality of honeybee larvae receiving tetracycline (Peng et al., 1992) and adult bumblebees receiving antibiotic mixtures (Koch

and Schmid-Hempel, 2011b) to direct toxic effects. However, the death of the treated workers could be due to an indirect toxic effect of the two antibiotics as the absence of a gut microbiota can cause a loss of appetite which in turn may lead to starvation. Two main mechanisms could underlie this. First, impaired pollen digestion could possibly result in gut obstruction and reduced nutrient uptake. Histochemical staining of embedded sections of the

Figure 4. Increase in the mean drone production $\pm$ SD ($n=10$; $P<0.05$) and mean mass of drones $\pm$ SD ($n=20$; $P<0.01$) in streptomycin-treated colonies

alimentary canal of honeybees showed that pollen wall components like hemicelluloses and pectic acids undergo partial digestion (Klungness and Peng, 1984), suggesting that the bacterial community assists pollen digestion. Indeed the genomes of honeybee and bumblebee do not encode pectinase (Weinstock et al., 2006; The bumblebees Genome Sequencing Consortium, unpublished data), while for instance gammaproteobacteria do (Engel et al., 2012). Second, the antibiotics treatment may have impaired the nutrient synthesis by (endo)symbiotic bacteria in the insect which in turn may have resulted in bumblebee fatigue. It has already been suggested that bacterial metabolites like short chain fatty acids act as molecules with nutritional value for bees (Martinson et al., 2011).

In microcolonies treated with streptomycin alone we observed opposite effects compared to treatment with the two antibiotics (S+T) together, as these performed better than the controls (Figure 4). Streptomycin is bactericidal mainly for gram-negative bacteria but also for some gram-positive ones, and therefore a shift in the bacterial community was to be expected. Since the bumblebee gut microbiota has already been characterized (Koch and Schmid-Hempel, 2011a), we can speculate about the effects of treatment with streptomycin. The gram-negative bacteria (i.e., gammaproteobacteria, betaproteobacteria, and bacterioidetes) that are normally present in

the bumblebee gut will have difficulties to grow, leaving a selective advantage for the gram-positive bacteria such as Firmicutes, *Lactobacillus*, and *Bifidobacteria*. Indeed, the microbiota in streptomycin-treated (S) bumblebees clusters separately from the untreated (C) ones (Figure 3a). The DGGE pattern of streptomycin-treated bumblebees mainly consists of *Lactobacillus* sp. However, it should be remarked that the fact that the treatment with streptomycin alone resulted in unique bands, does not mean that the representative bacteria are not present in the control samples. Indeed some queen-right colonies also harbored this specific *Lactobacillus* sp. (data not shown). We believe that the streptomycin treatment here created an environment for the *Lactobacillus* sp. to further colonize the gut, perhaps by spilling over from biofilms in the insect crop since many *Lactobacillus* species tend to reside in these environments (Vasquez et al., 2012). In order to get more insights into the colonization dynamics of *Lactobacillus* in the bumblebee gut, quantitative PCR of *Lactobacillus* sp. is needed to determine changes in bacterial load. Furthermore by fluorescence in situ hybridization with *Lactobacillus*-specific 16S rRNA probes, one can visualize the microhabitat of the targeted bacteria (Moter and Göbel, 2000).

If we look at the natural occurrence of the identified *Lactobacillus* sp. we saw a 100 % max

identity with *Lactobacillus* sp. previously identified in honeybees. Based on Babendreier et al. (2007) and Martinson et al. (2011), this bacteria belongs to the Firm-5 phylotype, which is reported as a phylotype specific for corbiculate bees (Martinson et al., 2011). Our specific sequence, KC477412, actually has not been detected in bumblebees before (Koch and Schmid-Hempel, 2011a; Koch et al., 2013). The closest matches are bumblebee gut bacterial sequences AJ971929 (97 % max identity) (Mohr and Tebbe, 2007), JQ388898, and JQ388899.1 (96 % max identity). On the contrary, in honeybees, the KC477412 is quite common (Martinson et al., 2011). We speculate that *Lactobacillus* sp. (KC477412) only occurs in low numbers in wild bumblebees and it got introduced in commercial bumblebees by their contact with honeybee collected pollen and further got established after streptomycin treatment.

When scoring the fitness of the microcolonies treated with streptomycin alone, it was interesting that these showed an improved fitness with higher drone numbers and higher drone masses. For humans, beneficial properties of lactic acid bacteria have been described as ingestion of lactic acid bacteria has been associated with a range of health benefits, including immune system modulation and increased resistance to malignancy and infectious illness (Soccol et al., 2010). In addition, *Lactobacillus* sp. is known to produce exopolysaccharides for biofilm formation to protect against invading “negative” bacteria (Vasquez et al., 2012). Vasquez et al. (2012) speculated that the potential health benefits of exopolysaccharides in humans could also apply for bees.

In conclusion, in this project we altered the gut microbiota of bumblebees (*B. terrestris*) by use of antibiotics, which in turn had effects on the fitness of these bumblebee workers. We mainly explain our results through beneficial effects by *Lactobacillus* sp. but the possibility also remains that the positive fitness effects are a consequence of the loss of “negative” bacteria. We used reared bumblebees, and although the three bacterial species identified were identical with those previously identified in wild bumblebees or honeybees, wild bumblebees probably have a

different microbial community composition. Therefore our results cannot be extrapolated toward wild bumblebees. Further research on these aspects of microbiological communities in the insect gut and insect–microbe interactions may open new innovative avenues in the mass-rearing of bumblebees used for the biological pollination in agriculture.

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Evaluation du mutualisme entre *Bombus terrestris* et son microbiote grâce à l'utilisation de micro-colonies

Interactions hôte-micro-organisme / pollinisateur / insecte social / *Bombus terrestris* / streptomycine / *Lactobacillus* / probiotique

Abschätzung des Mutualismus zwischen *Bombus terrestris* und seinen Mikrobioten durch Verwendung von Mikrokolonien

Wirt-Mikroben-Interaktionen / soziale Bestäuber / *Bombus terrestris* / Streptomycin / *Lactobacillus* / Probiota

REFERENCES

- Aizen, M.A., Garibaldi, L.A., Cunningham, S.A., Klein, A.M. (2008) Long-term global trends in crop yield and production reveal no current pollination shortage but increasing pollinator dependency. *Curr. Biol.* **18**(20), 1572–1575
- Babendreier, D., Joller, D., Romeis, J., Bigler, F., Widmer, F. (2007) Bacterial community structures in honeybee intestines and their response to two insecticidal proteins. *FEMS Microbiol. Ecol.* **59**(3), 600–610
- Bakke, I., De Schryver, P., Boon, N., Vadstein, O. (2011) PCR-based community structure studies of bacteria associated with eukaryotic organisms: A simple PCR strategy to avoid co-amplification of eukaryotic DNA. *J. Microbiol. Meth.* **84**(2), 349–351
- Breeuwer, J.A., Werren, J.H. (1990) Microorganisms associated with chromosome destruction and repro-

- ductive isolation between two insect species. *Nature* **346**(6284), 558–560
- Broderick, N.A., Raffa, K.F., Goodman, R.M., Handelsman, J. (2004) Census of the bacterial community of the gypsy moth larval midgut by using culturing and culture-independent methods. *Appl. Environ. Microbiol.* **70**(1), 293–300
- Brownlie, J.C., Johnson, K.N. (2009) Symbiont-mediated protection in insect hosts. *Trends Microbiol.* **17**(8), 348–354
- Chandler, J. A., Lang, J. M., Bhatnagar, S., Eisen, J. A., Kopp, A. (2011) Bacterial communities of diverse *Drosophila* species: Ecological context of a host-microbe model system. *PLoS Genetics* **7**(9) doi:10.1371/journal.pgen.1002272
- Clarke, K.R. (1993) Nonparametric multivariate analyses of changes in community structure. *Aust J Ecol* **18**(1), 117–143
- Clarke, K.R., Green, R.H. (1988) Statistical design and analysis for a biological effects study. *Mar. Ecol. Progr.* **46**(1–3), 213–226
- Corby-Harris, V., Pontaroli, A.C., Shimkets, L.J., Bennetzen, J.L., Habel, K.E., Promislow, D.E.L. (2007) Geographical distribution and diversity of bacteria associated with natural populations of *Drosophila melanogaster*. *Appl. Environ. Microbiol.* **73**(11), 3470–3479
- Dillon, R.J., Vennard, C.T., Buckling, A., Charnley, A.K. (2005) Diversity of locust gut bacteria protects against pathogen invasion. *Ecol. Lett.* **8**(12), 1291–1298
- Engel, P., Martinson, V.G., Moran, N.A. (2012) Functional diversity within the simple gut microbiota of the honey bee. *Proc. Natl. Acad. Sci. U S A* **109**(27), 11002–11007
- Forsgren, E., Olofsson, T.C., Vasquez, A., Fries, I. (2010) Novel lactic acid bacteria inhibiting *Paenibacillus* larvae in honey bee larvae. *Apidologie* **41**(1), 99–108
- Ghazoul, J. (2005) Buzziness as usual? Questioning the global pollination crisis. *Trends Ecol. Evol.* **20**(7), 367–373
- Klungness, L.M., Peng, Y.S. (1984) A histochemical study of pollen digestion in the alimentary canal of honeybees (*Apis mellifera* L.). *J. Insect Physiol.* **30**(7), 511–521
- Koch, H., Schmid-Hempel, P. (2011a) Bacterial communities in central European bumblebees: Low diversity and high specificity. *Microb. Ecol.* **62**(1), 121–133
- Koch, H., Schmid-Hempel, P. (2011b) Socially transmitted gut microbiota protect bumble bees against an intestinal parasite. *Proc. Natl. Acad. Sci. U S A* **108**(48), 19288–19292
- Koch, H., Abrol, D.P., Li, J., Schmid-Hempel, P. (2013) Diversity and evolutionary patterns of bacterial gut associates of corbiculate bees. *Mol. Ecol.* **22**(7), 2028–2044
- Martinson, V.G., Danforth, B.N., Minckley, R.L., Rueppell, O., Tingek, S., Moran, N.A. (2011) A simple and distinctive microbiota associated with honey bees and bumble bees. *Mol. Ecol.* **20**(3), 619–628
- Marzorati, M., Wittebolle, L., Boon, N., Daffonchio, D., Verstraete, W. (2008) How to get more out of molecular fingerprints: practical tools for microbial ecology. *Environ. Microbiol.* **10**(6), 1571–1581
- McKee, E.E., Ferguson, M., Bentley, A.T., Marks, T.A. (2006) Inhibition of mammalian mitochondrial protein synthesis by oxazolidinones. *Antimicrob. Agents Chemother.* **50**(6), 2042–2049
- Mertens, B., Boon, N., Verstraete, W. (2005) Stereospecific effect of hexachlorocyclohexane on activity and structure of soil methanotrophic communities. *Environ. Microbiol.* **7**(5), 660–669
- Mommaerts, V., Sterk, G., Smagghe, G. (2006) Hazards and uptake of chitin synthesis inhibitors in bumblebees *Bombus terrestris*. *Pest. Manag. Sci.* **62**(8), 752–758
- Moran, N.A., McCutcheon, J.P., Nakabachi, A. (2008) Genomics and evolution of heritable bacterial symbionts. *Annu. Rev. Genet.* **42**, 165–190
- Mohr, K.I., Tebbe, C.C. (2007) Field study results on the probability and risk of a horizontal gene transfer from transgenic herbicide-resistant oilseed rape pollen to gut bacteria of bees. *Appl. Microbiol. Biotechnol.* **75**(3), 573–582
- Moter, A., Göbel, U.B. (2000) Fluorescence in situ hybridization (FISH) for direct visualization of microorganisms. *J. Microbiol. Meth.* **41**(2), 85–112
- Peng, Y.S.C., Mussen, E., Fong, A., Montague, M.A., Tyler, T. (1992) Effects of chlortetracycline of honey-bee worker larvae reared *in vitro*. *J. Invert. Pathol.* **60**(2), 127–133
- Riegler, M., O'Neill, S.L. (2007) Evolutionary dynamics of insect symbiont associations. *Trends Ecol. Evol.* **22**(12), 625–627
- Socol, C.R., Vandenbergh, L.P.D., Spier, M.R., Medeiros, A.B.P., Yamaguchi, C.T., Lindner, J.D., Pandey, A., Thomaz-Socol, V. (2010) The potential of probiotics: a review. *Food. Tech. Biotechnol.* **48**(4), 413–434
- Steffan-Dewenter, I., Potts, S.G., Packer, L. (2005) Pollinator diversity and crop pollination services are at risk. *Trends Ecol. Evol.* **20**(12), 651–652
- Van de Wiele, T., Boon, N., Possemiers, S., Jacobs, H., Verstraete, W. (2004) Prebiotic effects of chicory inulin in the simulator of the human intestinal microbial ecosystem. *FEMS Microbiol. Ecol.* **51**(1), 143–153
- Vasquez, A., Forsgren, E., Fries, I., Paxton, R.J., Flaberg, E., Szekely, L., Olofsson, T.C. (2012) Symbionts as major modulators of insect health: Lactic acid bacteria and honeybees. *PLoS One* **7**(3), e33188. doi:10.1371/journal.pone.0033188
- Velthuis, H.H.W., van Doorn, A. (2006) A century of advances in bumblebee domestication and the economic and environmental aspects of its commercialization for pollination. *Apidologie* **37**(4), 421–451

- Vervaeen, H., Temmerman, R., Devos, L., Boon, N., Verstraete, W. (2006) Introduction of a boost of *Legionella pneumophila* into a stagnant-water model by heat treatment. *FEMS Microbiol. Ecol.* **58**(3), 583–592
- Weinstock, G.M., Robinson, G.E., Gibbs, R.A., Worley, K.C., Evans, J.D., et al. (2006) Insights into social insects from the genome of the honeybee *Apis mellifera*. *Nature* **443**(7114), 931–949
- Wilkinson, T.L. (1998) The elimination of intracellular microorganisms from insects: an analysis of antibiotic-treatment in the pea aphid (*Acyrtosiphon pisum*). *Comp. Biochem. Physiol. Mol. Integr. Physiol.* **119**(4), 871–881
- Wittebolle, L., Marzorati, M., Clement, L., Balloi, A., Daffonchio, D., Heylen, K., Vos, P.D., Verstraete, W., Boon, N. (2009) Initial community evenness favors functionality under selective stress. *Nature* **458**, 623–626