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Apidologie (2014) 45:467–477

DOI: 10.1007/s13592-013-0262-1

Original article

Density-dependent negative responses by bumblebees to bacteria isolated from flowers

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Received 7 June 2013 – Revised 1 October 2013 – Accepted 5 December 2013

Abstract – Flowers offer habitats for bacterial communities that are often characterized by low diversities but high densities. The composition of these communities and the dissemination of bacteria between flowers receive increasing attention, whereas the ecological functions of flower-associated but non-phytopathogenic bacteria remain understudied. We screened bacteria isolated from nectar, petals and leaves of two plant species for their potential to affect flower–visitor interactions. We took advantage of the proboscis extension reflex (PER) of bumblebees evoked by sugar and investigated whether bacteria associated with the reward may interrupt this reflex. Cultivated bacteria were transferred into a watery glucose solution in increasing densities and their effect on the proportion of bumblebees displaying the PER after antennal contact with glucose solutions and bacteria was scored. In all but one trial, the proportion of bumblebees that accepted the watery glucose solution was negatively correlated with the bacterial density. Nearly half of the bacteria tested evoked avoidance at naturally occurring densities. Our results suggest that bacteria colonizing flowers have the potential to negatively affect the reproduction of plants via reduced visits by pollinators.

aversion / Bacilli / *Bombus terrestris* / plant–bacteria–animal interactions / proboscis extension reflex

1. INTRODUCTION

Flowers are described as habitats for microorganisms that often reach high densities in nectar (Herrera et al. 2008; Fridman et al. 2012), on petals (Junker et al. 2011), stigmas (Huang et al. 2012) and pollen (Fuernkranz et al. 2012). These communities are mostly characterized by a low species richness (Alvarez-Perez et al. 2012; Alvarez-Perez and Herrera 2013) compared to microbial communities found on other plant surfaces (Krimm et al. 2005; Junker et al. 2011). The identities of bacteria in these communities differ between flower parts (Fuernkranz et al. 2012), plant parts (Junker et al. 2011), plant species (Fridman et al. 2012) and change with the age of flowers (Shade et al. 2013). Thus, floral nectar and other flower parts are inhabited by specific bacterial and fungal communities (Junker et al. 2011; Fridman et al. 2012; Alvarez-Perez and Herrera 2013), which may be shaped by species-specific characteristics of flowers and nectar (Alvarez-Perez et al. 2012; Pozo et al. 2012; Junker and Tholl 2013). Flower nectar is

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Manuscript editor: Bernd Grünewald

characterized by often high sugar concentrations, the presence of secondary metabolites and proteins that may reduce or prevent the proliferation of microorganisms (Adler 2000; Carter and Thornburg 2004). Furthermore, flower volatiles (terpenoids and aromatics) were shown to affect the bacterial communities found on petals and stigmas (Junker et al. 2011; Huang et al. 2012; Junker and Tholl 2013). According to the notion that floral characteristics select the microbial communities, yeast assemblages in nectar of two plant species were shown to be subsets of the communities colonizing insects and other plant parts in close proximity to nectar (Pozo et al. 2012). Alternatively to flower traits shaping communities, the presence of different flower visitors potentially disseminating bacteria and yeast may structure the microbial communities in nectar and other flower parts (compare to Brysch-Herzberg 2004). This assumption is supported by the demonstrated ability of insects and birds to transport bacteria and yeast between flowers either in context of biocontrol or in natural settings. Social and solitary bees have been found to effectively disseminate bacterial and fungal antagonists of plant pathogens between flowers (van der Steen et al. 2004; Maccagnani et al. 2009). In a natural habitat, the distribution of microfungi in flowers of *Mimulus aurantiacus* was independent of habitat and nectar parameters but was affected by the density of flowers and the location of flowers (Belisle et al. 2012). Furthermore, the same fungi as found in nectar were isolated from the tongues and beaks of hummingbirds, which are important pollinators of *M. aurantiacus* (Belisle et al. 2012). Lachance et al. (2001) found that differences in yeast communities in nectar are explainable by plant species and geography but also by the main flower visitors that carried distinct yeast species. These results suggest that flower visitors have a profound effect on microbial communities of nectar and flowers of naturally growing plant species.

While the knowledge about the distribution of bacteria and yeast on flower organs and their mode of dissemination increased in the last few years, the ecological functions of floral microorganisms remain largely unknown. Exceptions are studies on pathogens such as *Erwinia amylovora* (Buban et al. 2003) and their antagonists (Johnson et al. 1993), e.g. it has been shown that individual bacterial strains and defined communities of bacteria associated to flowers serve as biocontrol agents against plant diseases (Fuernkranz et al. 2012). However, few studies are reported on interactions between microorganisms, flowers and pollinators: Herrera et al. (2008) clearly demonstrated that yeasts strongly alter the sugar concentration and composition of nectar, which may affect plant reproduction via reduced pollinator activity. So far, only one study linked bacteria in nectar, pollinator behaviour and seed set of a plant species: Vannette et al. (2012) showed that the presence of the bacterium *Gluconobacter* sp. decreased nectar pH and sugar concentration of *M. aurantiacus*, which may have been the reason for reduced nectar consumption by hummingbirds and thus seed set. In contrast, yeast-containing nectar was more attractive to bumblebees but reduced the fecundity of flowers (Herrera et al. 2013). In a different study, bumblebees avoided consumption of sugar water contaminated with *Crithidia bombi*, a protozoon that parasitizes bumblebees, which was interpreted as 'prophylactic behaviour of bumblebees' (Fouks and Lattorff 2011). In contrast, the bumblebees readily consumed sugar water contaminated with *Escherichia coli* (strain JM109) (Fouks and Lattorff 2011).

These studies suggest that microorganisms may affect flower–visitor interactions, but further evidence is missing. Nonetheless, from other systems, it is well described that microbiomes alter the behaviour of animals and the outcome of interactions (Ezenwa et al. 2012; Davis et al. 2013). One remarkable example is given by Leroy et al. (2011) demonstrating that *Staphylococcus sciuri* bacteria that inhabit the honeydew of the aphid *Acyrthosiphon pisum* produce volatiles that attract the natural enemies of the aphids. Thus, further detailed studies on how microorganisms interfere with flower–visitor interactions and plant sexual reproduction may produce novel insights that may augment our view on floral ecology.

In this study, we screened a number of bacterial strains for their potential to affect flower–visitor interactions by experimentally

testing the ability of bumblebees to perceive and respond to bacteria. Bacteria were isolated from flowers and leaves of a bumblebee-visited plant species and from flowers of a plant species usually not visited by bumblebees. In the lab, we examined density-dependent effects of bacteria dissolved in sugar water on the proboscis extension reflex (PER) of bumblebees and subsequent consumption of contaminated resources.

2. MATERIAL AND METHODS

2.1. Cultivation and identification of bacteria isolated from flowers and leaves

We sampled flowers and leaves of *Lamium maculatum* (Lamiaceae, $n=15$) in the Botanical Garden of the Heinrich-Heine University Düsseldorf, Germany to isolate, cultivate and identify epiphytic bacteria associated with these surfaces and with floral nectar. The plant species was chosen because it is visited by bumblebees (Knuth 1908). Additionally, we sampled flowers of *Achillea millefolium* (Asteraceae, $n=3$), which is usually not visited by bumblebees (Knuth 1908). Bacteria from sources that are usually contacted by bumblebees (flowers and nectar of *L. maculatum*) and from sources that do not interact with bumblebees (leaves of *L. maculatum* and flowers of *A. millefolium*) were chosen to compare their effects on bumblebees' responses. Flower buds were individually marked, and opened flowers were sampled 3 days later. Sampling of leaves and flowers was performed using gloves, scissors and forceps sterilized with ethanol between each sampling step. Samples were transported to the lab in unused plastic bags.

Nectar was extracted from flowers using autoclaved micro-capillary tubes (5 μL) and transferred into 50 μL phosphate buffered saline (PBS, Sigma-Aldrich, Steinheim, Germany). Likewise, flowers and leaves were placed into 100 μL PBS and sonicated for 7 min. Afterwards, 15 μL (for nectar samples) or 45 μL (for flower and leaf samples) of the PBS containing epiphytic bacteria was streaked out on autoclaved (20 min, 125 °C) LB agar medium (LB-Medium Powder, AppliChem, Darmstadt, Germany; Bacto Agar, Becton, Dickinson and Company, Sparks, USA), a medium previously used to cultivate bacteria from leaves and flowers (Krimm et al. 2005; Junker et al. 2011). Fungicide (Cycloheximide, Sigma-Aldrich, Steinheim, Germany, 30 μg l^{-1}) was added to LB medium to prevent fungi from growing. After an incubation for 72 h at room temperature, colony forming units (cfu) were distinguished based on colour, shape and texture and one colony per distinct morphotype was cultivated on separate LB agar plates containing no fungicide resulting in 125 strains individually cultivated. For our screening for potential effects of bacteria on bumblebee behaviour, we haphazardly chose 13 strains of bacteria isolated from *L. maculatum* and nine from *A. millefolium*. Bacteria used for identification and for experiments thus originated from a single cfu.

The 22 chosen bacterial strains were identified via 16S rRNA genes. DNA of one colony per strain was extracted using the High Pure PCR Template Preparation kit (Roche, Grenzach-Wyhlen, Germany) following the manufacturer's instructions. For the subsequent polymerase chain reaction (PCR), we used 23 μL Mastermix 16S Basic, DNA free (Molzym, Bremen, Germany) including the primer pair 27f and 1492r (see Junker et al. 2011) and added 2 μL DNA template. As a negative control, we added 2 μL DNA-free PCR water instead of the template. A thermocycler (Eppendorf Mastercycler gradient, Hamburg, Germany) with the following programme was used: initial denaturation at 94 °C for 3 min, 35 cycles of 94 °C for 30 s, 52 °C for 30 s, 72 °C for 100 s and a final extension step at 72 °C for 5 min. PCR products were purified using the PCR-purification kit (Seqlab, Göttingen, Germany) according to the manufacturer's instructions. DNA concentration was measured using a NanoDrop-ND-1000 (NanoDrop Technologies, Wilmington, USA), and a total of 375 ng of DNA along with 1 μL primer 27f (20 pmol) and 1 μL 10 mMol Tris were sent to Seqlab (Göttingen, Germany) for Extended HotShot sequencing. Sequences were quality start- and end-trimmed according to phred scores with 4Peaks (Nucleobytes, Amsterdam, NL) or even discarded in case of low phred scores. Further, ChimeraSlayer (http://microbiomeutil.sourceforge.net) was used for detection of chimeric amplification artefacts, which were not detectable in our sequences. Sequences were taxonomically assigned to the lowest taxonomical level possible by using the Ribosomal Database Project (RDP) Classifier (Wang et al. 2007) and analysed with Krona Tools (Ondov et al. 2011).

2.2. Bioassay with *Bombus terrestris*

To test whether bumblebees (*Bombus terrestris*, biobest, Westerlo, Belgium, purchased from re-nature, Dorsten, Germany, $n=8$ colonies) perceive and consume bacteria, we took advantage of the PER following contact to a sugary solution with the antennae (Anfora et al. 2011). Bumblebees were reared in the lab and were fed with sucrose solution and pollen. Prior to the tests, bumblebees did not have contact to flowers or glucose solution. Individual workers were not used twice for the same test but potentially in trials testing the response to a different bacterial strain. We used 2 ml Eppendorf tubes and removed the tip creating a hole large enough for the head of bumblebees but small enough to restrain them inside the tube. Once bumblebees entered the tube, we fixated them using foam plugs. Prior to experiments, bumblebees were starved for 3 h. About 40 bacterial colonies were picked from agar plates, put into 1.5 ml watery glucose solution (20 %) and the mix was sonicated for 7 min resulting in a homogenous distribution of bacteria in the solution. A dilution series was prepared resulting in six solutions with decreasing densities of bacteria; watery glucose solution (20 %) not contaminated with bacteria was used as control. Strips of filter paper were soaked with one of the different solutions each. To reveal the density of bacteria in each of the dilutions (number of bacteria μL^{-1}), we transferred 1 μL of the two solutions with the lowest densities of bacteria on LB agar plates and streaked them out. Three days later, the number of cfu's was counted and the number of bacteria was extrapolated to the solutions with higher densities of bacteria. This dilution series resulted in a mean±SD density of 321±265 cfu μL^{-1} (minimum 7 cfu μL^{-1}) in the lowest concentration and 82,839±67,299 cfu μL^{-1} (max 204,595 cfu μL^{-1}) in the highest concentration. Thus, bacteria were presented in a range of densities that overlaps with densities found on floral surfaces (Junker et al. 2011) and nectar (Fridman et al. 2012).

The principle procedure was the same in each of the experiments. We allowed bumblebees to make contact with their antennae to filter papers soaked with either the control solution or one of the solutions containing bacteria. The experimental solutions were offered in order of increasing density of bacteria. The first and the last as well as in between each of the experimental solutions, a control solution (20 % glucose, no bacteria) was offered testing for the bumblebees' motivation to consume sugar water. Thus, each bumblebee worker was tested with 13 solutions. Per bacterium $n=12$ bumblebees were tested. If bumblebees extended their proboscis, the same solution that touched the antenna was offered to the proboscis. Presence or absence of proboscis extension after antenna contact with the solution and consumption of the solution was noted.

To test whether contamination with bacteria is perceived by antenna and/or proboscis, the experiments with three selected strains of bacteria were repeated, but this time, antennae were touched with sugar solution only and solutions containing bacteria were offered for consumption, i.e. bumblebees had contact to bacteria with their proboscis, only.

To test whether living bacteria affect the PER or whether alterations of the solution by bacteria (e.g. consumption or alteration of sugar, reductions in pH, metabolites of bacteria) influence the behaviour of bumblebees, the same experiment was repeated with three bacterial strains that had the strongest effect in the previous experiment. However, prior to the experiments, the solutions containing bacteria were incubated for 1 h at room temperature (which was the time we needed for other experiments) and afterwards autoclaved.

Microorganisms are known to change the sugar composition in nectars (Herrera et al. 2008; Vannette et al. 2012), which may affect the behaviour of flower visitors. We performed PER experiments by offering watery solutions containing glucose, sucrose or fructose (20 %) to test whether bumblebees respond differently to the sugars. Each of the 12 bumblebees tested were allowed to respond five times to each of the sugars.

Apart from the conversion of the sugars, microorganisms also decrease total sugar concentration of nectar (Herrera et al. 2008; Vannette et al. 2012). To test whether PER is affected by different concentrations of glucose, the experiment was repeated with watery solutions containing 10, 15 or 20 % of glucose but no bacteria. Each bumblebee worker ($n=15$) was tested five times with the concentration series starting with lowest concentration. Proportion of trials that

resulted in PER and consumption of glucose solution per concentration was calculated for each bumblebee.

2.3. Statistical analysis

The PER experiments resulted in binary data with either the bumblebees extended their proboscis and consumed the reward (1) or they did not (0). To test whether an increasing density of bacteria resulted in a changing proportion of bumblebees that consume the solution, we performed generalized linear models (GLMs) with binomial error distribution and logit link function. We used the bumblebee's responses (0 or 1) as response variable and the density of bacteria (cfu μL^{-1}) as explanatory variable. This model was compared to a neutral model containing no explanatory variable using an analysis of deviance (Crawley 2005). If both models are significantly different from each other, the explanatory variable has an effect on the bumblebee's responses, i.e. the proportion of bumblebees extending their proboscis and/or consuming the solutions is dependent on bacterial density.

Additional to the GLMs, we estimated nonlinear least squares of the parameters a and b of logistic functions $r = \frac{e^{a+bd}}{1+e^{a+bd}}$ with r=proportion of bumblebees displaying PER at a given density of bacteria in the solution and d=density of bacteria, describing binary responses and reflecting the logit link functions used for the GLMs (Crawley 2005). For each bacterium tested, we used the estimates of a and b and set r=0.5 (i.e. half of the bumblebees displayed PER and consumed the solution) to calculate the density of bacteria d that is required to prevent 50 % of bumblebees from consuming the solution. All statistical analyses were performed with the statistical computing software R (R Development Core Team 2011).

3. RESULTS

Of the 22 haphazardly chosen bacterial strains, 11 were classified to the Bacilli class distributed across the genera *Bacillus*, *Falsibacillus*, *Salirhabdus* and two unidentified Bacilli bacteria. Two further bacteria were identified to belong to the Actinobacteria (*Microbacterium* and an unidentified Micrococcineae bacterium) and one to the Alphaproteobacteria (*Sphingomonas*), respectively. The taxonomic status of eight bacteria remained unclear (Table I, EMBL Nucleotide Sequence Database accession numbers for sequences: HF912247–HF912260).

All but one bacterial strain tested evoked density-dependent responses by the bumblebees (see results of GLMs, Table I), i.e. the proportion of bumblebees that accepted the watery glucose solution (PER and consumption) was negatively correlated to the density of bacteria (cfu μL^{-1}) in the solution (see results of logistic regression, Table I and Figure 1). We made the observation that bumblebees that displayed the PER after antennal contact to the solutions always consumed the solution, too. Although nearly all bacteria caused bumblebees to refuse the consumption of the watery glucose solution at certain densities, we found a broad range of densities that were required to cause 50 % of bumblebees (r=0.5) to reject the reward. The average density at r=0.5 was 87,292± 76,383 cfu μL^{-1} (mean±SD), the minimum density needed was 499 cfu μL^{-1}, the maximum 195,819 cfu μL^{-1}, nine times it was below or close to 31,000 cfu μL^{-1} (Table I), a density that has been found in nectar sampled in the field (Fridman et al. 2012). Densities of bacteria associated to leaves were significantly lower at 50 % rejection rate by bumblebees (11,728± 4,533 cfu μL^{-1}) than bacteria associated to flowers (110,905±70,318 cfu μL^{-1}; Mann–Whitney U-test P=0.032). However, the bacterium that elicited negative responses at the lowest density (Bacilli, 499 cfu μL^{-1} at r=0.5) was isolated from nectar. On average, bacteria isolated from *L. maculatum* flowers were avoided in lower densities (69,323± 63,895 cfu μL^{-1}) than those associated to *A. millefolium* flowers (152,488±48,460 cfu μL^{-1}; Mann–Whitney U-test P=0.028). The decreasing proportion of bumblebees accepting the reward in each of the test sequences was not the result of a

Table I. Bacteria used for experiments with bumblebees. Taxonomic status of bacteria along with the bootstrap (BS) support of the RDP classifier (Wang et al. 2007), and their origin are given. For each bacterium, the results of the generalized linear model and the logistic regression are given testing for the density dependence of the bumblebee's responses. Additionally, the estimated density of bacteria (cfu μL^{-1}) at r=0.5, i.e. the density of bacteria that prevents 50 % of bumblebees to consume the watery glucose solution, is given. Densities below or close to that what was found in floral nectar in the field (Fridman et al. 2012) are highlighted in bold.

Number	Class (BS support)	Order (BS support)	Genus (BS Support)	Plant species	Plant organ	GLM with binomial error distribution					Logistic regression		
						Deviance	Total deviance	df_1	df_2	p	r^2	p	density of bacteria at r=0.5
1	Bacilli (100)	Bacillales (100)	*Bacillus* (90)	*L. maculatum*	Flower	39.13	63.11	1	155	<0.001	0.49	<0.001	**7,943**
2	Actinobacteria (100)	Micrococcineae (100)	*Microbacterium* (100)	*L. maculatum*	Flower	27.64	63.11	1	155	<0.001	0.29	<0.001	109,056
3	Bacilli (100)	Bacillales (100)	*Salirhabdus* (59)	*L. maculatum*	Flower	40.10	74.29	1	155	<0.001	0.43	<0.001	110,087
4	NA	NA	Unidentified Bacteria	*L. maculatum*	Flower	26.55	50.86	1	155	<0.001	0.33	<0.001	**2,081**
5	NA	NA	Unidentified Bacteria	*L. maculatum*	Flower	45.01	119.48	1	155	<0.001	0.41	<0.001	185,171
6	NA	NA	Unidentified Bacteria	*L. maculatum*	Flower	20.76	44.24	1	155	<0.001	0.26	<0.001	108,429
7	Actinobacteria (87)	Micrococcineae (76)	Unidentified Micrococcineae	*L. maculatum*	Flower	52.72	111.58	1	155	<0.001	0.48	<0.001	**31,317**
8	Bacilli (100)	Bacillales (100)	*Bacillus* (85)	*L. maculatum*	Leaf	20.76	44.24	1	155	<0.001	0.26	<0.001	**10,550**
9	Bacilli (100)	Bacillales (100)	*Bacillus* (94)	*L. maculatum*	Leaf	66.24	94.21	1	155	<0.001	0.72	<0.001	**12,011**
10	Alphaproteobacteria (100)	Sphingomonadales (100)	*Sphingomonas* (100)	*L. maculatum*	Leaf	26.55	50.86	1	155	<0.001	0.33	<0.001	**3,944**
11	NA	NA	Unidentified Bacteria	*L. maculatum*	Leaf	39.36	123.26	1	155	<0.001	0.35	<0.001	**17,297**
12	NA	NA	Unidentified Bacteria	*L. maculatum*	Leaf	27.94	44.24	1	155	<0.001	0.40	<0.001	**14,838**
13	Bacilli (75)	NA	Unidentified Bacilli	*L. maculatum*	Nectar	44.86	149.41	1	129	<0.001	0.49	<0.001	**499**
14	Bacilli (100)	Bacillales (100)	*Bacillus* (99)	*A. millefolium*	Flower	17.73	50.86	1	155	<0.001	0.21	<0.001	149,645
15	Bacilli (100)	Bacillales (100)	*Falsibacillus* (79)	*A. millefolium*	Flower	5.21	12.09	1	155	0.023	0.08	<0.001	57,391
16	Bacilli (100)	Bacillales (99)	*Salirhabdus* (50)	*A. millefolium*	Flower	18.74	57.14	1	155	<0.001	0.19	<0.001	195,819
17	Bacilli (100)	Bacillales (100)	*Salirhabdus* (65)	*A. millefolium*	Flower	26.55	50.86	1	155	<0.001	0.33	<0.001	178,767
18	Bacilli (100)	Bacillales (100)	*Salirhabdus* (73)	*A. millefolium*	Flower	10.59	21.40	1	155	<0.001	0.16	<0.001	167,905
19	Bacilli (56)	NA	Unidentified Bacilli	*A. millefolium*	Flower	16.15	29.65	1	155	<0.001	0.24	<0.001	191,167
20	NA	NA	Unidentified Bacteria	*A. millefolium*	Flower	20.76	44.24	1	155	<0.001	0.26	<0.001	190,109
21	NA	NA	Unidentified Bacteria	*A. millefolium*	Flower	32.91	63.11	1	155	<0.001	0.38	<0.001	89,099
22	NA	NA	Unidentified Bacteria	*A. millefolium*	Flower	10.59	21.40	1	155	<0.01	0.04	0.99	NA

decreasing motivation of the bumblebees to consume glucose. This was indicated by the result that in most trials bumblebees consumed watery glucose solution containing no bacteria (13th position of the test sequence) after the solution with the highest density of bacteria had been tested (12th position of the test sequence, Figure 2).

For experiments were bumblebees had contact to bacteria with their proboscis only and were the PER was triggered with a watery glucose solution containing no bacteria, we chose three bacterial strains that were refused by the bumblebees in comparatively low densities in previous experiments (Table I, *Bacillus* sp. #1, unidentified bacterium #12 and unidentified *Bacilli* bacterium #13). In these experiments, the bumblebees consumed each watery glucose solution regardless the density of bacteria that was in the same range as in the previous experiment. Therefore, the response was $r=1$ (display of PER and consumption of solution) in all of the trials. The lack of variation in the response variable prevented the use of GLMs and nonlinear regression.

Likewise, watery glucose solution in which the same bacteria (see above) had been incubated for 1 h and subsequently had been autoclaved did not negatively affect responses by bumblebees. Regardless the identity and density of bacteria, bumblebees displayed PER and consumed the solution, i.e. $r=1$ in all trials. Thus, the use of statistic was prevented by the lack of variance in the response variable, too.

Bumblebees displayed PER and consumed the sugar solution in most cases after their antenna contacted watery solution containing glucose (97 % of positive responses), sucrose (97 %) or fructose (95 %). Thus, we were not able to detect any differences in the acceptance of the different sugars (Friedman rank sum test $Chi^2=0.5$, $df=2$, $P=0.78$). Furthermore, proportion of bumblebees displaying the PER and consuming sugar solution was unaffected by the glucose concentration of the watery solution (proportion=0.77±0.05 (mean±SE), Friedman rank sum test $Chi^2=0.08$, $df=2$, $P=0.96$).

4. DISCUSSION

Above-ground plant parts are one of the largest habitats for microorganisms that colonize these structures in high diversities and abundances (Lindow and Brandl 2003; Fridman et al. 2012). Accordingly, flowers, nectar and leaves of *L. maculatum* and *A. millefolium* were colonized by a variety of cultivatable bacteria. Half of the haphazardly selected strains that we used for our experiments belong to the Bacilli, a bacterial class that is commonly found on plant surfaces (Ercolani 1991; Fuernkranz et al. 2012), and representatives of the genus *Bacillus* are frequent but most often non-pathogenic inhabitants of guts of insects (Jensen et al. 2003). Interestingly, the microbiome associated with honeybee surfaces and guts as well as their stored food was found to be dominated by Bacilli (Mattila et al. 2012).

Our experiments clearly demonstrated that bumblebees are able to perceive bacteria associated to flowers and nectar and to avoid the consumption of resources contaminated with the microorganisms. These responses were density-dependent, i.e. bumblebees tolerated bacteria in certain doses but the proportion of individuals rejecting the rewards increased above a bacteria strain-specific threshold in all but one bacterial strain. Four out of eight bacterial strains isolated from *L. maculatum* flowers and all of the bacteria isolated from leaves of this species elicited negative responses of bumblebees at densities that may be realistically expected under natural conditions (compare to Junker et al. 2011; Fridman et al. 2012). Bacteria associated to flowers of *A. millefolium* were required in much higher densities to elicit similar responses. The negative effect on bumblebee behaviour was not affected by the taxonomic affiliation of the bacteria, i.e. bacterial strains avoided by bumblebees in natural concentration are scattered within the phylogeny. Thus, our finding in combination with the results of Vannette et al. (2012) may suggest that floral bacteria potentially interfere with pollination via negative effects on the behaviour of insect pollinators.

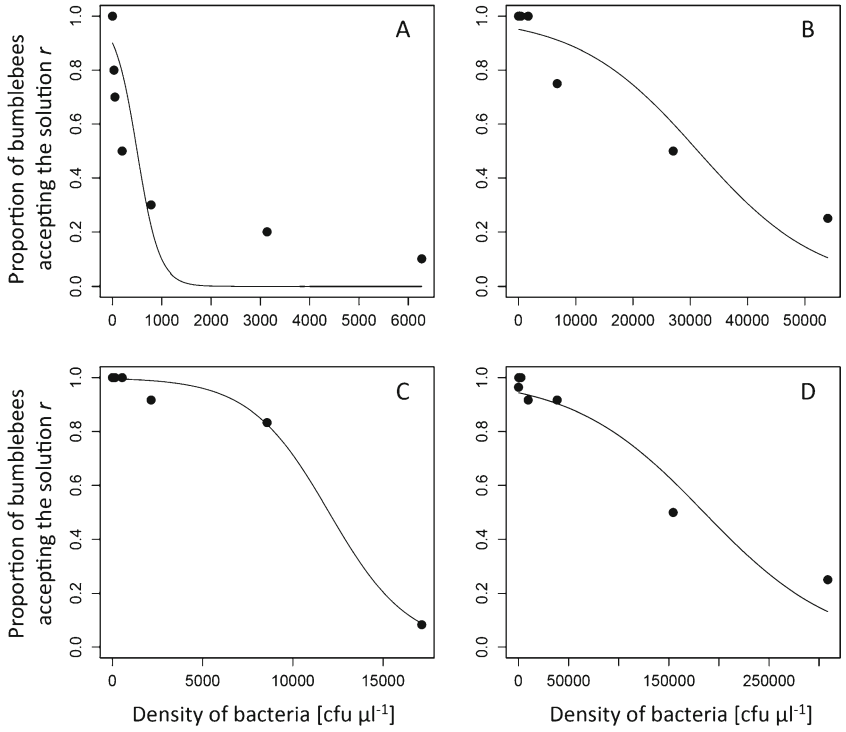

Figure 1. Proportion of bumblebees accepting watery glucose solution containing variable densities of selected bacteria (**a** unidentified Bacilli bacterium (Table I #13), **b** Micrococcineae (#7), **c** *Bacillus* sp. (#9) and **d** unknown bacteria (#5)). Regression line represents the logistic function based on binary data, i.e. either bumblebees displayed proboscis extension reflex and consumed the reward (1) or not (0). Note the different scales on the *x*-axis.

Nectar and pollen foraging bees probe the resources with their antenna and also have antennal contact to other flower parts including the petals (Lunau et al. 2009; Evangelista et al. 2010) and thereby gathering gustatory, olfactory and tactile information (Haupt 2004). This information may be used to evaluate the palatability and quality of their food. In our experiments, the bumblebees always consumed the offered reward once they extended their proboscis after antennal contact to the filter paper soaked with sugar (and acceptable densities of bacteria). If the bumblebees judged the resource as unpalatable due to contamination with bacteria, they did not extend their proboscis. Therefore, because bumblebees touch floral surfaces such as petals during landing and consumption of nectar (Evangelista et al. 2010), we may assume that bumblebees avoid flowers with bacterial contamination on any parts of the flowers not only if the rewards are spoiled.

Nonetheless, note that our selection of bacteria was biased by the cultivation of bacteria (which was essential for the experiments) as only a small proportion of bacteria associated with plant surfaces is cultivatable on standard media (Yang et al. 2001). Therefore, our experiments show the general potential of bacteria to negatively interfere with pollinator visits, but interpretation about the commonness and frequency of these effects under natural conditions is limited. Future studies should consider the whole bacterial communities to assess the probably more diverse functions of bacteria in the interaction with flowers and their visitors.

While our results show that bumblebees refuse to consume rewards after they perceived

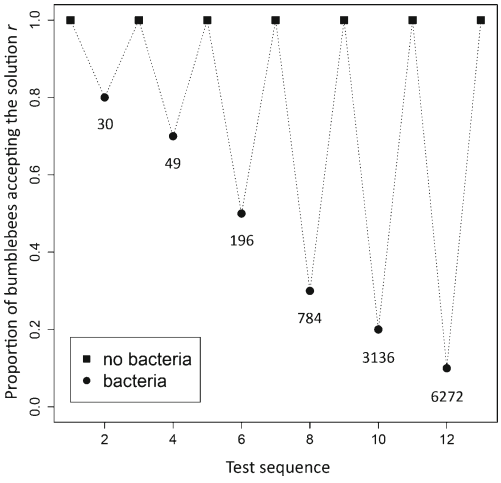

Figure 2. Test sequence of the experiment using an unidentified Bacilli bacterium (Table I #13). Bumblebees were allowed to alternately respond to watery glucose solution (closed squares) and watery glucose solution containing bacteria (closed circles) to control whether a decreasing proportion of bumblebees accepting the reward is resulting from bacterial contamination or from a decreasing motivation of the bumblebees. Numbers below *circles* denote the density of bacteria (cfu μL^{-1}) at each trial within the test sequence. After the last trial using the highest density of bacteria (r=0.1), all bumblebees displayed the PER (r=1) indicating a persistent motivation to consume glucose.

the presence of bacteria in sufficient density, the experiments do not inform about the mechanism responsible for the avoidance. However, we are able to exclude the reduction of the sugar concentration and the alteration of the sugar composition by bacteria as potential reasons for the rejection of the sugar solution because in our bioassay, the bumblebees did not discriminate between concentrations and types of sugar. Thus, although bacteria and other microorganisms had been shown to reduce the sugar concentration of nectar and to alter the composition of sugars (Herrera et al. 2008; Vannette et al. 2012), these effects may not be sufficient to significantly affect the behaviour of flower visitors. Future work should therefore focus on cues that are perceived by flower visitors and that may mediate the avoidance behaviour. Potential cues are a low pH (Vannette et al. 2012), contamination of sugar water with bacterial metabolites or the detection of living bacterial cells. The latter two hypotheses are supported by our results suggesting that the presence of living (not dead) bacteria can be detected with the antenna but not with the proboscis. However, note that avoidance of unsuitable resources is attenuated if bumblebees are harnessed in experimental trials in the lab (Sanchez 2011), potentially explaining the consumption of the contaminated sugar water once the bumblebees extended their proboscis. We have increasing knowledge about the effects of bacterial volatiles on animal behaviour (Kai et al. 2009; Davis et al. 2013). The examples reported in the literature indicate that the scents emitted by bacteria mediate either attraction of insects to hosts and oviposition sites (Ponnusamy et al. 2008; Leroy et al. 2011) or repellence from contaminated resources (Stensmyr et al. 2012), and the diversity of volatiles synthesized by bacteria (Schulz and Dickschat 2007) suggests potential further effects.

Given the commonness and the diversity of bacteria colonizing plant surfaces (Ercolani 1991) including flower surfaces and nectar (Junker et al. 2011; Fridman et al. 2012) that may be associated with negative effects on pollinator behaviour and thus potentially on plant reproduction (compare to Vannette et al. 2012), selection may favour flowers with defence mechanisms against bacteria that interfere with pollination. Bees need antennal contact to flower surfaces in order to initiate landing (Evangelista et al. 2010) and thus may sense the presence of bacteria on petals, not only in nectar. Therefore, defence mechanisms may be expected on all parts of flowers and may not be restricted to the rewards for pollinators (Junker and Tholl 2013). In concordance with this hypothesis, bacterial communities were found to be non-randomly composed across plant species, both in nectar (Alvarez-Perez and Herrera 2013) and on petals (Junker et al. 2011) suggesting habitat filtering due to antimicrobial flower traits. This notion may be supported by our result that bacteria isolated from leaves evoked a avoidance behaviour in bumblebees at lower densities than those isolated from flowers, which may suggest that the flowers investigated successfully inhibited the growth of the most detrimental bacteria regarding pollinator visits.

We conclude that flower visitors are able to perceive a considerable proportion of bacteria in relevant densities that colonize flowers, which may elicit an avoidance behaviour at strain-specific densities. As shown by Vannette et al. (2012), this may translate into reduced seed set in plants. These negative effects by bacteria may explain why flowers have various defence mechanisms against bacteria that may structure floral microbiomes potentially by inhibiting the growth of the most detrimental strains (Junker and Tholl 2013). Nonetheless, flower–bacteria–animal interactions are not understood in enough detail and the ecological and evolutionary significance of these interactions remains largely unknown, which should be addressed in future work.

ACKNOWLEDGMENTS

We thank Christina Loewel for helpful advices and Karl Köhrer for technical support. The project was supported by the Deutsche Forschungsgemeinschaft (DFG, JU 2856/1-1).

Réponses négatives densité-dépendantes des bourdons aux bactéries isolées à partir des fleurs

Aversion / bactérie / *Bombus terrestris* / interactions plante–bactérie–animal / PER / réflexe d'extension du proboscis

Hummeln vermeiden Bakterien auf Blüten in natürlichen Dichten

Abneigung / Bacilli / *Bombus terrestris* / Pflanzen–Bakterien–Tier Interaktionen / Rüsselreflex

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