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INVITED ORIGINAL RESEARCH PAPER

Epiphytic bacteria on lettuce affect the feeding behavior of an invasive pest slug

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Abstract
Plant–animal interactions are not isolated pairwise relationships but are always accompanied by diverse assemblages of microbes. Additional to direct effects of microorganisms on their hosts, recent investigations demonstrated that bacteria associated with plants can modify the behavior of organisms of higher trophic levels. However, in the context of herbivory, functions of non-phytopathogenic bacteria colonizing leaf surfaces remain understudied. This study showed that naturally occurring epiphytic bacteria affect the feeding behavior of a generalist herbivore. Epiphytic bacteria isolated from leaves of *Lactuca sativa* var. *capitata* were screened for their potential to influence feeding choices of the slug *Arion vulgaris*. Cultivated bacteria were inoculated in artificial food substrates or on sterile leaves of gnotobiotic lettuce plants and were offered to slugs in different behavioral bioassays. A large proportion of bacterial strains tested induced behavioral alterations in the feeding choices of slugs. Behavioral responses of slugs were further modified by antibiotic treatment of slugs prior to choice tests indicating that both bacteria associated with plants and animals affect plant–animal interactions. Our results emphasize the important role of bacteria in plant–animal interactions and suggest a prominent role of bacteria in herbivory in natural, horticultural, and agricultural systems.

Keywords
Arion vulgaris; dual choice experiment; herbivory; microorganisms; plant–bacteria–animal interactions

Introduction

Plant–animal interactions, such as pollination and herbivory, are important ecosystem processes and are usually investigated in isolation. However, plant–animal interactions cannot be considered as isolated pairwise relationships only, since they rather take place in complex communities [1]. Next to further plant and animal species that may affect the quantity, quality and outcome of pairwise interactions [2–4], microorganisms associated with plants and animals may also interfere with these interactions [5–8]. From both natural and managed ecosystems, it is well known that plants harbor complex and diverse microbiomes specific for each plant microenvironment [9,10] such as the anthosphere [11], endosphere [12], rhizosphere [13], and phyllosphere [14]. Recently, it has been shown that microbes colonizing petals [9,11], stigmata [10,15], pollen [16], and nectar [10,17,18] clearly outnumber eukaryotic flower visitors and also affect pollinator behavior [6,8] and reproduction of plants [19,20]. For endophytes, it is already well established that interactions between a plant host and its microbes can evoke alteration in the host by inducing gene expression of plants'

defensive metabolic pathways [21]. Even plant growth promoting rhizobacteria have been recorded to affect aboveground herbivore damage by modifying the plant defensive enzymes [22]. The phyllosphere hosts, next to algae, fungi, and yeasts, bacterial communities that are the predominant and the most abundant epiphytes on leaves [14,23–25], which can reach, for example on lettuce leaves, abundances between 10^6 and 10^7 colony-forming units (cfus) per g tissue [23,24,26]. Bacterial community composition is shaped by biotic and abiotic parameters such as the availability of nutrients and the presence of growth inhibiting substances [13,25,27]. Resident microbes significantly contribute to the regulation of further incoming, transient colonizers such as pathogens [28–30]. Furthermore, it is well established that epiphytic bacteria have profound effects on plant wellness, biomass gain, and reproduction, either positive or negative [31,32]. However, how these bacteria mediate interactions between plants and herbivores remains unknown, albeit it is well established that bacteria within the crop, digestive glands and salivary glands of herbivorous slugs contribute to digestion such as cellulose degradation [33]. Furthermore, the intake of different diets has been shown to affect the natural gut microbiota of snails [34]. An understanding of the ecology of microbial communities in the phyllosphere interacting among each other, with their host and other organisms may extend our view on their impact on multitrophic interactions [6,35]. This knowledge may further yield new approaches in applied ecology, for instance, in integrated pest management for crop protection or in farming practices [1,26,35].

In this study, we performed several dual-choice bioassays to examine the potential effects of bacterial communities associated with leaves on the behavior of herbivorous organisms. Epiphytic bacterial communities of *Lactuca sativa* var. *capitata* plants were isolated and identified by 16 S rRNA gene sequencing. These strains were then used in different bioassays to evaluate their effect on feeding choices of the herbivorous pest slug *Arion vulgaris*. *Lactuca sativa* L. is a crop with worldwide economic importance [26,36], and an increasing human consumption over the last recent years [37]. This agronomical important plant species was chosen because it is one of the most heavily injured crops damaged by *A. vulgaris*, which is among the 100 worst invasive alien species in Europe causing dramatic losses in *L. sativa* [38,39].

We used bioassays offering food substrates or sterile lettuce leaves inoculated with individual or multiple bacterial strains isolated from lettuce leaves to slugs to test the following predictions:

- Food choices of a generalist herbivore (*A. vulgaris*) are affected by individual bacterial strains and by multistrain assemblages.
- Bacterial communities associated with the slugs' digestive system (experimentally modified by antibiotic treatment of slugs) influence the preferences for and aversions against individual bacterial strains and multistrain assemblages.

Given the importance of bacteria in slugs' digestive system [33], the impoverishment of symbiotic bacteria after antibiotic treatment may influence the slugs' behavior and thus feeding choices. Our study emphasizes the necessity to consider bacteria in plant–animal interactions for a full understanding of such processes in natural and agricultural systems.

Material and methods

Bacteria isolated from lettuce leaf surfaces

For the collection, isolation, and cultivation of leaf-associated epiphytic bacteria, seedlings of *Lactuca sativa* (Gardenline, Germany) were established from seeds in the greenhouse. After 3 weeks, seedlings were relocated to a semi-natural field site within the botanical garden of the University of Salzburg for 1 week to allow colonization by natural bacterial communities. For the sampling of bacteria, single leaves from 10 plant individuals were collected. Sampling was done using sterile forceps to prevent contaminations, and each leaf was stored in a separate tube containing 5 mL autoclaved phosphate buffered saline (PBS tablet, Sigma-Aldrich R, Germany). To extract

the epiphytic bacteria, tubes containing leaves were sonicated for 7 min (following the standard procedures based on [9] and [14]). 100 µL of a 10^{-2} dilution of sonicated PBS, containing epiphytic bacteria, were streaked out on autoclaved (120°C for 35 min) R2A agar medium (Sigma-Aldrich R, Germany). Cycloheximide (Sigma-Aldrich R, Germany; 30 mg L^{-1}) was added to prevent the growth of fungi. After an incubation period of 72 h, emerging colony-forming units (cfus) of different morphotypes were selected according to differences in color, size, and appearance. One colony per distinct morphotype was then cultivated on autoclaved LB agar medium (Panreac AppliChem, Germany) supplemented with Agar Bacteriology grade (Panreac AppliChem, Germany) and 1 g L^{-1} D-(+)-glucose (Sigma-Aldrich R, Germany) without fungicide. To identify the bacterial isolates, the region coding for the 16S rRNA was sequenced. A PCR was performed by using the Promega GoTaq R G2 DNA Kit (Germany) according to the manufacturer's instruction. DNA of isolated colonies was transferred to 29 µL of the mastermix, containing the primers ALer1_341f CCT ACG GGA GGC AGC AG and Buniv_907r CCG TCA ATT CMT TTG AGT TT (Metabion, Germany) and Taq polymerase (Promega, Germany). As a negative control, 1 µL DNA-free PCR water was added instead of the template. The PCR (Gene Amp R PCR System 9700, Applied Biosystems) was run with one cycle for initial denaturation at 94°C for 3 min followed by 25 cycles of 30 s at 94°C, 30 s at 52°C, and 1 min 20 s at 72°C, with a final step of 7 min at 72°C and subsequently cooldown to 4°C. DNA concentrations (ng/µL) and the spectra of wavelengths of nucleid acids were quantified and controlled by a microvolume UV-Vis spectrophotometer NanoDrop 2000 (Peqlab Biotechnology GmbH, Germany) by using 2 µL of each extracted and purified PCR product. The minimum concentration for sequencing was 30 ng/µL^{-1}. 15 µL of each DNA sample were mixed with 2 µL primer (ALer1_341f) (100 pm/µL). For DNA sequencing, all DNA samples were sent to MWG-Biotech AG, Germany. Sequences were taxonomically assigned to the lowest level possible by comparing to sequences at the GenBank nucleotide database [40].

Gnotobiotic lettuce plants

In order to test whether our results are replicable under more natural conditions, we tested the effect of one bacterial strain associated with fresh plant material on the feeding choices of slugs. *Lactuca sativa* plants were cultivated on MS nutrient medium including vitamins and 0.7% plant agar (both from Duchefa, the Netherlands) and supplemented with 1% sucrose. 300 mL of medium was filled into autoclaved microboxes (191 × 185 × 185 H × B mm, 5000 mL, Combiness, Belgium). Two strips of a rayon sealing film (82.6 × 142.9 mm) (Area Seal Film TM BS-25, Excel Scientific, USA) were placed on the adhesive top of the solidified medium to absorb the remaining liquid and to provide the isolation of appearing epicotyls from the medium. *Lactuca sativa* seeds were surface sterilized according to the vapor-phase protocol [41,42] to exclude contaminations of seed associated bacterial communities colonizing seedlings during the germination process [14,43]. 2.5 mL reaction tubes containing seeds (lid halfopen allowing fumes to enter the tubes) were placed in a desiccator. A beaker containing 100 mL of bleach (sodium hypochlorite 50% solution, NaClO, VWRR, Germany) was added to the desiccator. 3.3 mL of hydrochloric acid 37% (HCl) were added to the bleach for chlorine gas development. After sterilization overnight, the desiccator was left open for 20 min. The reaction tubes containing the sterilized seeds were gently removed and also left open for 1 hour until the remaining gas was evaporated. Seeds were stratified for 1 week (at 4°C) before being placed on the growth medium. Plants were grown in the laboratory (18°C, 60% relative humidity, 12 h light/dark cycle)

Food choice experiment

Arion vulgaris individuals (body size ranging 2.6 to 4.8 cm) were collected in the field and kept individually in sterile air-permeable microboxes (40 × 80 mm H × W, 210 mL, Combiness, Belgium) under 12 h light/dark cycle in the lab with air conditioned

environment (18°C, 60% relative humidity). Slugs were fed every 2–3 days with 0.5 g artificial food substrate (modified after [33,44]) prior to the experiments. The food substrate consisted of 300 g grinded *L. sativa* leaves, 400 mL H_2O, 10 g bran, and 8 g LB-medium and was stored at −20°C. Before usage, it was supplemented with Agar Bacteriology grade after defrosting, autoclaved (120°C for 35 min), and offered to slugs after hardening. Half of the slugs were fed with a food substrate additionally containing the antibiotics chlortetracycline hydrochloride and chloramphenicol (Sigma-Aldrich R, Germany), in order to test whether bacteria associated with the slugs' digestive system affect feeding choices. Both antibiotics were added to food substrates during cooling after autoclaving (500 μg g^{-1} each). The animals were starved for 24 h prior to the bioassays. Individual slugs were not reused for bioassays of the same or other treatments. Eight single strains of bacteria (*Arthrobacter* sp., *Bacillus cereus*, *B. licheniformis*, *B. megaterium*, *B. thuringiensis*, *B. subtilis*, *Brevundimonas* sp., *Fictibacillus* sp.) isolated from lettuce leaf surfaces were used in the experiments. Food substrate was inoculated with natural densities (cfus per mass freshweight, i.e., 4×10^5 and 5×10^6 cfus per g of tissue). Bacteria growing in LB-plates were transferred into 1 mL liquid LB-medium and optical densities (OD_{600}) of suspension were measured in a plate reader (ELX 808, Biotek Instruments, Germany). Based on these OD_{600} measurements, the volume of the suspension was determined and used to inoculate the food substrate. Additional to the inoculation with single bacterial strains, assemblages of four strains (*Arthrobacter* sp., *Bacillus cereus*, *B. licheniformis*, *B. megaterium* and *B. thuringiensis*, *B. subtilis*, *Brevundimonas* sp., *Fictibacillus* sp.) or assemblages of all eight strains were applied to the food substrate. Densities of bacterial assemblages were adjusted to the same densities as in trials with individual strains. For bioassays with sterile lettuce leaves, *Bacillus licheniformis* was chosen because it is one of the strains that evoked strong effects in experiments with artificial food substrates. Choice bioassays were conducted under sterile conditions in autoclaved (120°C for 35 min) containers (191 × 185 × 185 mm H × B , 5000 mL, Combiness, Belgium), where two food substrates (~1 g each, exact weight was measured prior to the tests) or lettuce leaves of different treatments were placed randomly on two opposite sides of the container. Sterile substrate/leaf was used as control, and the other substrate/leaf contained either a single bacterial strain or an assemblage of four or eight different strains. In bioassays with sterile plant material, the petioles of lettuce leaves were watered with wet sterile filter paper. Sterile aluminum foil prevented slugs from feeding on filter papers. Single slug individuals were placed in containers and were allowed to feed on the substrates overnight for 10 hours. Per strain and assemblage, $n = 12$ slug individuals previously treated with antibiotics (antibiotic-treated slugs) and $n = 12$ slugs without antibiotic treatment (control slugs) were tested. At the end of the experiment, both food substrates were removed from the containers and weighed again to the nearest centigram. Due to differences in leaf weights, we were not able to provide sterile lettuce leaves of a standardized mass in bioassays, therefore we recorded the slugs' position on either of the offered leaves (sterile or inoculated with *Bacillus licheniformis*) every 10 min for a total of 120 min. Slugs that did not touch either of the leaves were excluded from statistical analysis.

Statistical analysis

To test for preferences or aversions of control and antibiotic-treated slugs for food substrates inoculated with bacteria compared to sterile food substrates, the mass of consumed food substrates (bacteria-inoculated and sterile) was standardized between 0 and 1, with 0 as the lowest mass of consumed food substrate (bacteria-inoculated or sterile) and 1 as the highest mass of consumed food substrate. For each slug, the differences of standardized consumption of food substrates (bacteria-inoculated – sterile) were calculated. Negative differences thus show an aversion of slugs against bacteria, positive differences a preference. To test whether the calculated differences of the $n = 12$ control or antibiotics-treated slugs deviate from 0, a one-sample t test was performed for each trial. An additional Welch's t test was performed to test for different responses of antibiotic-treated slugs and control slugs towards strains or assemblages of strains. Additionally, analysis of variance (ANOVA) was performed, with

the standardized differences of consumption as the dependent variable and with slug treatment (antibiotic-treated slugs vs. control slugs) (Slug) and the strain of bacteria (Strain), or bacteria assemblages (Assemblage) as well as the two-way interaction as explanatory variable. To test the specific responses by each slug group towards sterile lettuce leaves inoculated with or without bacteria, standardized differences of slug visitation frequencies were calculated as described before. Statistical analysis was conducted as described for food substrates. All analyses were performed with the statistical computing software R 3.3.0 [45].

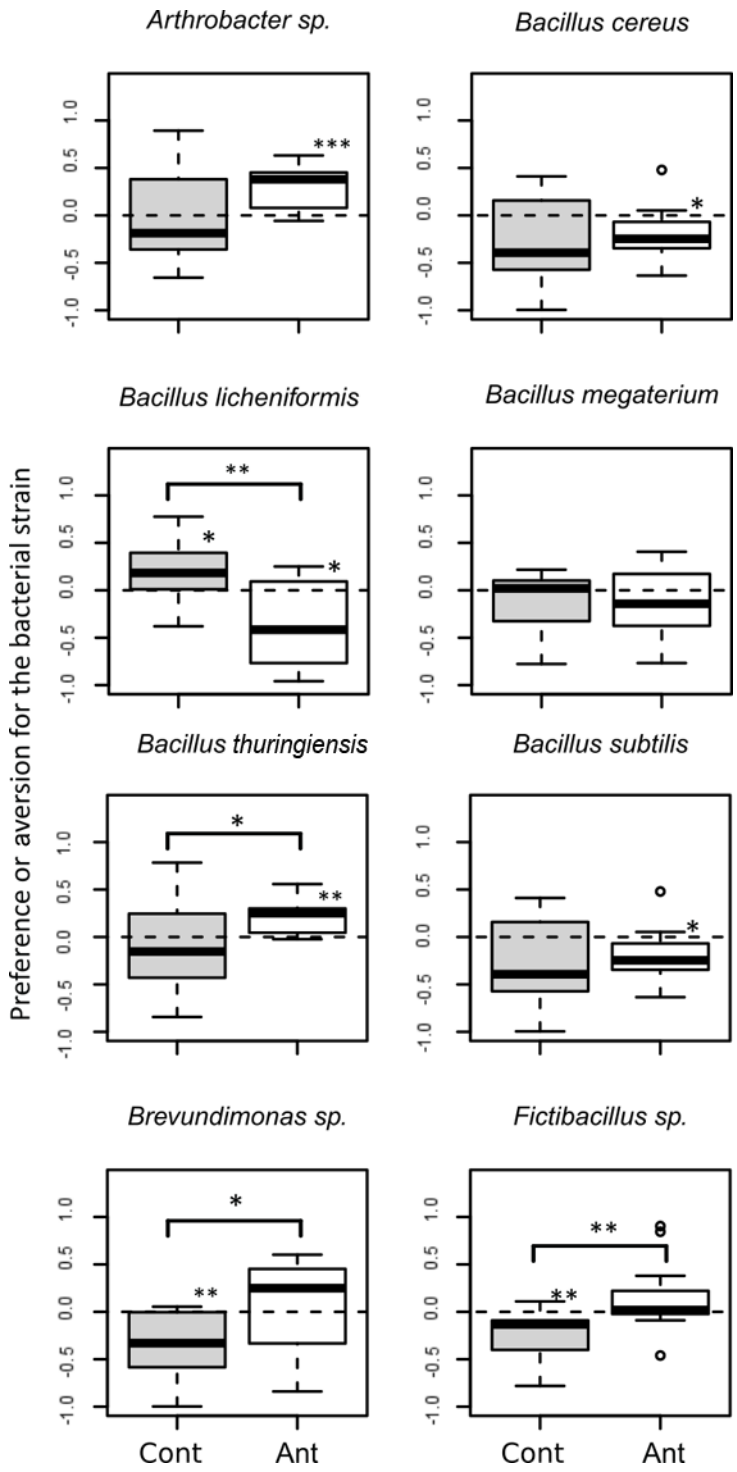

Fig. 1 Behavioral responses of *A. vulgaris* without antibiotic treatment $n = 12$ (Cont, grey boxplots) and treated with antibiotics $n = 12$ (Ant, white boxplots) to different bacterial strains. The differences of standardized consumption of food substrates (bacteria-inoculated – sterile) are shown. Negative differences thus show an aversion of slugs against bacterial strains, positive differences a preference. Data were analyzed with one sample *t* test. Significant preference or avoidance behaviors of slugs are indicated with asterisks $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. Black bars above the boxplots show significant differences in responses of control and antibiotic treated slugs analyzed by a Welch's *t* test.

Results

Experiments with artificial food

Individual bacterial strains inoculated in food substrates evoked either preferences or aversions compared to the sterile substrates (Fig. 1). Often, antibiotic treated slugs differently responded to the bacterial strains compared to the control slugs (Fig. 1). The strain of bacteria (bacterial strain), but not the slug treatment, had a significant effect on the differences of slug consumption (Tab. 1). However, the significant effect of two-way interaction between slug treatment and bacterial strain indicates that different bacterial strains evoke contrasting responses by both control and antibiotic-treated slugs (Tab. 1). Similar to single strains, slugs, either treated with antibiotics or untreated slugs, mostly responded differently to bacterial assemblages (Fig. 2). However, differences between bacteria-inoculated and control substrates were not as pronounced as compared to individual strains (Fig. 1, Fig. 2). Overall, slug treatment tendentially affected food choices (marginally significant), whereas identity of bacteria in assemblages was of minor importance (Tab. 2).

Experiments with sterile lettuce leaves

For bioassays with sterile lettuce leaves, we chose *Bacillus licheniformis*, one of the strains that evoked strong effects in previous experiments. Slugs showed no significant responses towards food substrates (Fig. 3; but note the small sample size of responding slugs: 4 for control slugs and 7 for antibiotic-treated slugs). However, the slugs' behavior was comparable to trials with artificial food substrates.

Tab. 1 Results of two-way interaction ANOVA with standardized differences in consumption of food substrate inoculated with bacteria and sterile food substrates as dependent variable and slug treatment (control or antibiotic-treated), bacterial strains, and their interaction as explanatory variable.

	df	*F*	*p*
Slug treatment	1	3.538	0.054
Bacterial strain	7	17.324	0.024**
Slug : Strain	7	13.156	<0.001***
Residuals	352		

Asterisks indicate significant effects (**$p < 0.01$ and ***$p < 0.001$) for explanatory variables and the two-way interaction.

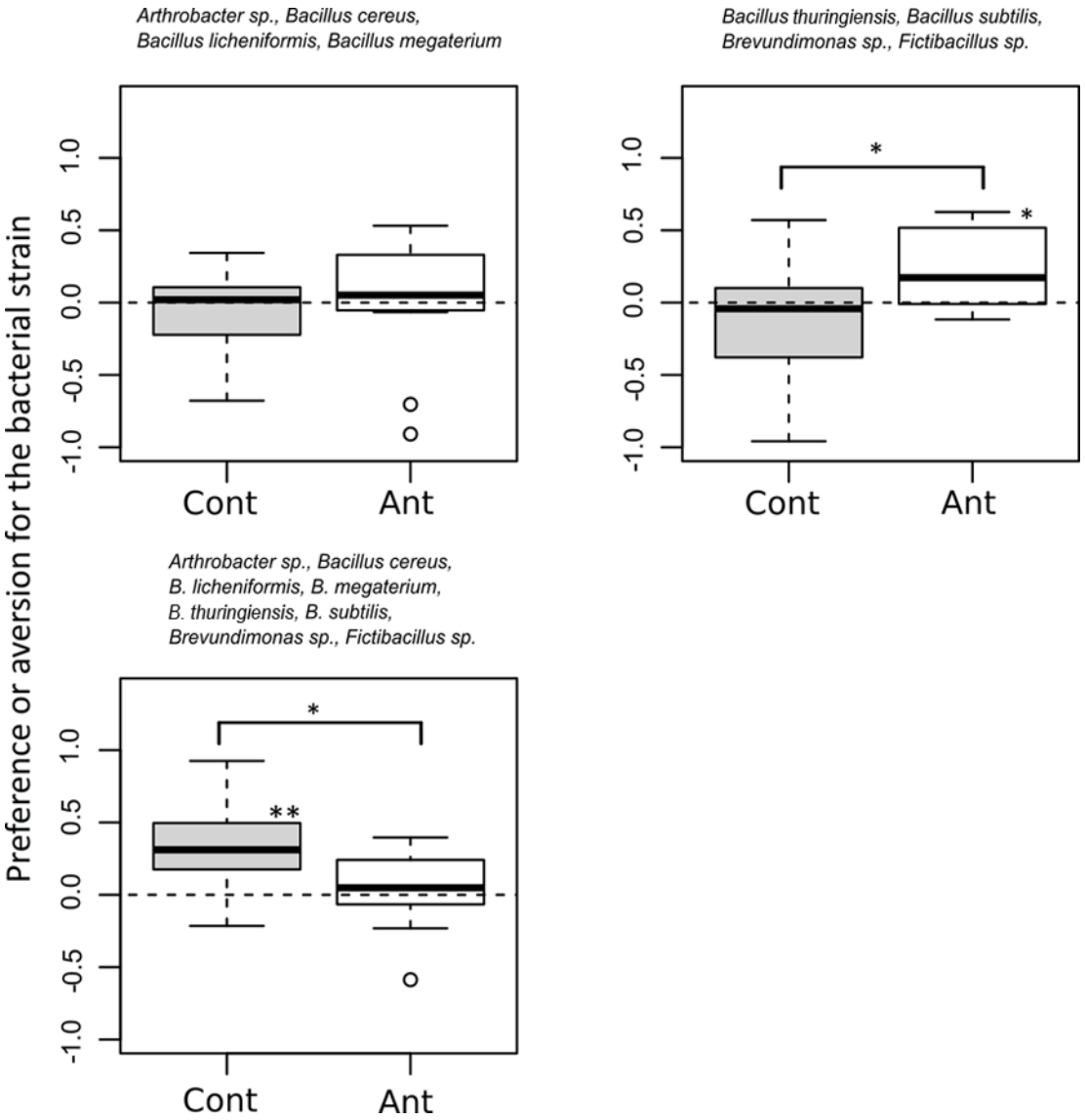

Fig. 2 Behavioral responses of *A. vulgaris* without antibiotic treatment $n = 12$ (Cont, grey boxplots) and treated with antibiotics $n = 12$ (Ant, white boxplots) to two assemblages of four prior selected bacterial strains. The differences of standardized consumption of food substrates (bacteria-inoculated – sterile) are shown. Negative differences thus show an aversion of slugs for bacteria, positive differences a preference. Data were analyzed with one sample *t* test. Significant preference or avoidance behaviors of slugs are indicated with asterisks *$p < 0.05$, **$p < 0.01$. Black bars above the boxplots show significant differences in responses of control and antibiotic treated slugs analyzed by a Welch's *t* test.

Tab. 2 Results of two-way interaction ANOVA with standardized differences in consumption of food substrate inoculated with bacterial assemblages and sterile food substrates as dependent variable and slug treatment (control or antibiotic-treated), bacterial strains, and their interaction as explanatory variable.

	df	*F*	*p*
Slug treatment (Slug)	1	8.277	<0.057
Bacterial assemblage	2	2.298	0.810
Slug : Assemblage	2	1.551	0.370
Residuals	124		

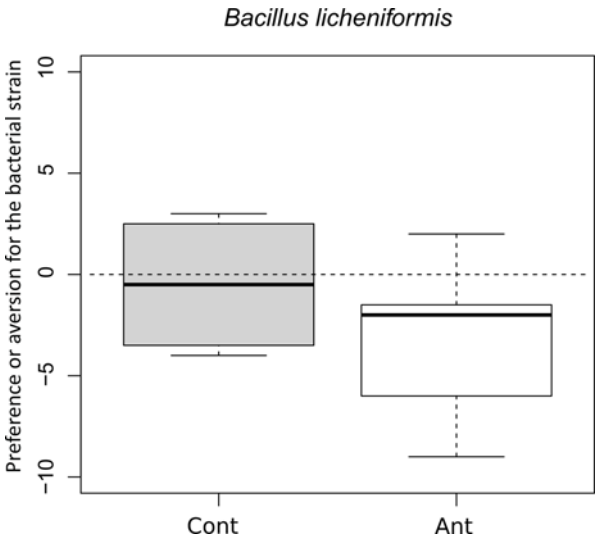

Fig. 3 Behavioral response of *A. vulgaris* individuals treated without antibiotic treatment $n = 4$ (Cont, grey boxplots) and with antibiotics $n = 7$ (Ant, white boxplots) to lettuce leaves inoculated with *Bacillus licheniformis*. The differences of slug visitation frequencies are shown. Visitations were recorded every 10 min over a duration of 120 min. Negative differences (numbers <0.0) thus show an aversion of slugs for bacteria, positive differences a preference (numbers >0.0). Data were analyzed with one sample *t* test and two-sample *t* test. No significant preference or avoidance behaviors were recorded.

Discussion

Our results indicate that distinct bacterial strains and assemblages of several strains, naturally colonizing the phyllosphere of lettuce *Lactuca sativa*, affect the feeding choices of the herbivorous slug *A. vulgaris*, either positively or negatively. The variable responses to different bacterial strains and assemblages of several strains suggest that feeding choices of slugs are dependent on the presence of specific bacterial strains. Additionally to bacterial strains associated with food items, bacterial communities colonizing the animals' digestive system seem to modify the behavior of slugs and thus feeding choices. Slugs treated with antibiotics often showed different behaviors than control slugs with natural gut bacterial communities. This suggests that there are complex interactions of the status of intrinsic (gut) and the composition of extrinsic (food) bacterial communities. Bacterial populations within the crop, digestive glands, and salivary glands of slugs have been considered to contribute to digestive processes like cellulose degradation [33]. Further, it was shown that the natural gut microbiota of snails can be altered by different diets [34]. Thus, our results, in combination with published results [33,34], suggest that in the absence of symbiotic bacteria, slugs might try to re-establish a diverse community of gut microbiota by food consumption.

In our study, assemblages of four or eight bacterial strains associated with food substrates evoked some preferences in slugs compared to sterile food substrates. For instance, antibiotic-treated slugs preferred food substrates with bacterial assemblages of *Bacillus thuringiensis*, *B. subtilis*, *Brevundimonas* sp., *Fictibacillus* sp. over sterile controls. By comparing these results with evaluations on single bacterial strains, where *Brevundimonas* sp. and *Fictibacillus* sp. had no effect on food choice, *Bacillus thuringiensis* was preferred, and *B. subtilis* was avoided, we can assume that certain bacterial strains might have a stronger influence on slug feeding choices than others; in the above-mentioned example, the attractiveness of *B. thuringiensis* might be superior to the deterring effect of *B. subtilis*. Other cases, where bacterial assemblages were neither preferred nor avoided by slugs, despite of clear effects of single strains on the food choice of slugs, suggest that effects of different strains offered in assemblage might cancel each other out. Thus, while our study clearly demonstrates the effect of individual bacterial strains and species-poor bacterial communities on feeding choices of animals, future studies are clearly required to understand additive or synergistic effects of a large number of strains. Note that our results are, due to methodological constraints, restricted to cultivatable bacteria representing only a small fraction of the diversity of bacteria associated with leaf surfaces. The phyllosphere is known to be colonized by highly diverse bacterial communities

rather than by single bacterial strains [10]. Considering bacterial epiphytes in the lettuce phyllosphere, Proteobacteria, Firmicutes, Actinobacteria, and Bacteroidetes are the most abundant phyla [26,29]. Additionally, variations of bacterial communities in the lettuce phyllosphere are a function of time, space, and environment [26,46]. Thus, while assemblages of only two bacterial strains will help to gain insights on the mechanisms involved on how individual strains interact to influence the feeding behavior of herbivores, future studies should also involve experiments with a higher and more natural bacterial diversity. Furthermore, future studies are required to reveal the mechanisms underlying bacterial effects on herbivores' behavior. For instance, the effects of epiphytic bacterial volatiles on multitrophic interactions [27,47] and potential physiological, metabolic, and genetic mechanisms driving these interactions need to be considered and should be combined with experimental manipulations of plant–bacteria–animal interactions in the field [2].

Potential future application

The continuously increasing amounts of pesticides and antibiotics used in agriculture and horticulture pose a threat to human health, the environment, biodiversity, and, consequently, to ecosystem functions and services [48,49]. By manipulating the natural bacterial community on the leaves of crop plants, supporting the presence/absence and abundance of bacterial strains (which deter herbivorous pests), it might be possible to reduce the application of pesticides in the future. By taking advantage of priority effects, favoring early colonizers in later successional stages of the community associated with substrates [50], it might be possible to inoculate pest-deterring natural bacterial strains on seedlings of crop plants, which will then become more resistant to pests. Whereas such applications of bacteria as biocontrol agents are so far not realized, our results clearly show the potential of the utilization of bacteria in horticultural and agricultural systems to reduce both the loss of crop and the amount of pesticides applied to fields.

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References

1. Christian N, Whitaker BK, Clay K. Microbiomes: unifying animal and plant systems through the lens of community ecology theory. Front Microbiol. 2015;6:869. https://doi.org/10.3389/fmicb.2015.00869
2. van Dam NM, Heil M. Multitrophic interactions below and above ground: en route to the next level. J Ecol. 2011;99(1):77–88. https://doi.org/10.1111/j.1365-2745.2010.01761.x
3. Poveda K, Steffan-Dewenter I, Scheu S, Tscharntke T. Effects of decomposers and herbivores on plant performance and aboveground plant–insect interactions. Oikos. 2005(108):503–510. https://doi.org/10.1111/j.0030-1299.2005.13664.x
4. van der Putten WH. A multitrophic perspective on functioning and evolution of facilitation in plant communities. J Ecol. 2009;97(6):1131–1138. https://doi.org/10.1111/j.1365-2745.2009.01561.x
5. Ezenwa VO, Gerardo NM, Inouye DW, Medina M, Xavier JB. Microbiology. Animal behavior and the microbiome. Science. 2012;338(6104):198–199. https://doi.org/10.1126/science.1227412
6. Junker RR, Romeike T, Keller A, Langen D. Density-dependent negative responses

by bumblebees to bacteria isolated from flowers. Apidologie. 2014;45(4):467–477. https://doi.org/10.1007/s13592-013-0262-1

7. Theis KR, Schmidt TM, Holekamp KE. Evidence for a bacterial mechanism for group-specific social odors among hyenas. Sci Rep. 2012;2:615. https://doi.org/10.1038/srep00615
8. Vannette RL, Gauthier ML, Fukami T. Nectar bacteria, but not yeast, weaken a plant–pollinator mutualism. Proc R Soc B. 2013;280:20122601. https://doi.org/10.1098/rspb.2012.2601
9. Junker RR, Loewel C, Gross R, Dötterl S, Keller A, Blüthgen N. Composition of epiphytic bacterial communities differs on petals and leaves. Plant Biol. 2011;13(6):918–924. https://doi.org/10.1111/j.1438-8677.2011.00454.x
10. Junker RR, Keller A. Microhabitat heterogeneity across leaves and flower organs promotes bacterial diversity. FEMS Microbiol Ecol. 2015;91(9):fiv097. https://doi.org/10.1093/femsec/fiv097
11. Shade A, McManus PS, Handelsman J. Unexpected diversity during community succession in the apple flower microbiome. MBio. 2013(4):e00602-00612. https://doi.org/10.1128/mbio.00602-12
12. Compant S, Clément C, Sessitsch A. Plant growth-promoting bacteria in the rhizo- and endosphere of plants: their role, colonization, mechanisms involved and prospects for utilization. Soil Biology and Biochemistry. 2010;42(5):669–678. https://doi.org/10.1016/j.soilbio.2009.11.024
13. Berendsen RL, Pieterse CM, Bakker PA. The rhizosphere microbiome and plant health. Trends Plant Sci. 2012(17):478–486. https://doi.org/10.1016/j.tplants.2012.04.001
14. Hirano SS, Upper CD. Bacteria in the leaf ecosystem with emphasis on *Pseudomonas syringae* – a pathogen, ice nucleus, and epiphyte. Microbiol Mol Biol Rev. 2000;64(3):624–653. https://doi.org/10.1128/MMBR.64.3.624-653.2000
15. Huang M, Sanchez-Moreiras AM, Abel C, Sohrabi R, Lee S, Gershenzon J, et al. The major volatile organic compound emitted from *Arabidopsis thaliana* flowers, the sesquiterpene (*E*)-b-caryophyllene, is a defense against a bacterial pathogen. New Phytol. 2012(193):997–1008. https://doi.org/10.1111/j.1469-8137.2011.04001.x
16. Fuernkranz M, Lukesch B, Müller H, Huss H, Grube M, Berg G. Microbial diversity inside pumpkins: microhabitat-specific communities display a high antagonistic potential against phytopathogens. Microb Ecol. 2012(63):418–428. https://doi.org/10.1007/s00248-011-9942-4
17. Fridman S, Izhaki I, Gerchman Y, Halpern M. Bacterial communities in floral nectar. Environ Microbiol Rep. 2012(4):97–104. https://doi.org/10.1111/j.1758-2229.2011.00309.x
18. Herrera CM, Garcia IM, Perez R. Invisible floral larcenies: microbial communities degrade floral nectar of bumble bee-pollinated plants. Ecology. 2008;89(9):2369–2376. https://doi.org/10.1890/08-0241.1
19. Eisikowitch D, Lachance MA, Kevan PG, Willis S, Collinsthompson DL. The effect of the natural assemblage of microorganisms and selected strains of the yeast *Metschnikowia reukaufii* in controlling the germination of pollen of the common milkweed *Asclepias syriaca*. Can J Bot. 1990(68):1163–1165. https://doi.org/10.1139/b90-147
20. Herrera CM, Pozo MI, Medrano M. Yeasts in nectar of an early-blooming herb: sought by bumble bees, detrimental to plant fecundity. Ecology. 2013(94):273–279. https://doi.org/10.1890/12-0595.1
21. Zahoor AW, Nasheeman A, Tabasum M, Riyaz-Ul-Hassan S. Plant–endophyte symbiosis, an ecological perspective. Appl Microbiol Biotechnol. 2015;99:2955–2965. https://doi.org/10.1007/s00253-015-6487-3
22. Pineda A, Zheng SJ, van Loon JJA, Pieterse CMJ, Dicke M. Helping plants to deal with insects: the role of beneficial soil-borne microbes. Trends Plant Sci. 2010;15(9):507–514. https://doi.org/10.1016/j.tplants.2010.05.007
23. Lindow SE, Brandl MT. Microbiology of the phyllosphere. Appl Environ Microbiol. 2003;69(4):1875–1883. https://doi.org/10.1128/AEM.69.4.1875-1883.2003
24. Mercier J, Lindow SE. Role of leaf surface sugars in colonization of plants by bacterial epiphytes. Appl Environ Microbiol. 2000;66(1):369–374. https://doi.org/10.1128/AEM.66.1.369-374.2000
25. Whipps JM, Hand P, Pink D, Bending GD. Phyllosphere microbiology with special

reference to diversity and plant genotype. J Appl Microbiol. 2008;105(6):1744–1755. https://doi.org/10.1111/j.1365-2672.2008.03906.x

26. Rastogi G, Sbodio A, Tech JJ, Suslow TV, Coaker GL, Leveau JH. Leaf microbiota in an agroecosystem: spatiotemporal variation in bacterial community composition on field-grown lettuce. ISME J. 2012;6(10):1812–1822. https://doi.org/10.1038/ismej.2012.32
27. Junker RR, Tholl D. Volatile organic compound mediated interactions at the plant–microbe interface. J Chem Ecol. 2013;39:810–825. https://doi.org/10.1007/s10886-013-0325-9
28. Hunter PJ, Hand P, Pink D, Whipps JM, Bending GD. Both leaf properties and microbe-microbe interactions influence within-species variation in bacterial population diversity and structure in the lettuce (*Lactuca* species) phyllosphere. Appl Environ Microbiol. 2010;76(24):8117–8125. https://doi.org/10.1128/AEM.01321-10
29. Williams TR, Moyne AL, Harris LJ, Marco ML. Season, irrigation, leaf age, and *Escherichia coli* inoculation influence the bacterial diversity in the lettuce phyllosphere. PLoS One. 2013;8(7):e68642. https://doi.org/10.1371/journal.pone.0068642
30. Jacques MA, Kinkel LL, Morris CE. Population sizes, immigration, and growth of epiphytic bacteria on leaves of different ages and positions of field-grown endive (*Cichorium endivia* var. *latifolia*). Appl Environ Microbiol. 1995;61(3):889–906.
31. Bulgarelli D, Schlaeppi K, Spaepen S, Ver Loren van Themaat E, Schulze-Lefert P. Structure and functions of the bacterial microbiota of plants. Annu Rev Plant Biol. 2013;64:807–838. https://doi.org/10.1146/annurev-arplant-050312-120106
32. Vorholt JA. Microbial life in the phyllosphere. Nat Rev Microbiol. 2012(10):828–840. https://doi.org/10.1038/nrmicro2910
33. Walker AJ, Glen DM, Shewry PR. Bacteria associated with the digestive system of the slug *Deroceras reticulatum* are not required for protein digestion. Soil Biology and Biochemistry. 1999;31:1387–1394. https://doi.org/10.1016/S0038-0717(99)00054-1
34. Cardoso AM, Cavalcante JJV, Vieira RP, Lima JL, Grieco MAB, Clementino MM, et al. Gut bacterial communities in the giant land snail *Achatina fulica* and their modification by sugarcane-based diet. PLoS One. 2012;7(3):e33440. https://doi.org/10.1371/journal.pone.0033440
35. Newton AC, Gravouil C, Fountaine JM. Managing the ecology of foliar pathogens: ecological tolerance in crops. Ann Appl Biol. 2010;157(3):343–359. https://doi.org/10.1111/j.1744-7348.2010.00437.x
36. FAO. FAOSTAT – crops – lettuce and chicory [Internet]. 2017 [cited 2017 Mar 24]. Available from: http://faostat.fao.org
37. Li Z, Zhao X, Sandhu AK, Gu L. Effects of exogenous abscisic acid on yield, antioxidant capacities, and phytochemical contents of greenhouse grown lettuces. J Agric Food Chem. 2010(26):6503–6509. https://doi.org/10.1021/jf1006962
38. Gismervik K, Bruheim T, Rarvik LM, Haukeland S, Skaar I. Invasive slug populations (*Arion vulgaris*) as potential vectors for *Clostridium botulinum*. Acta Vet Scand. 2014;56(65):1–7. https://doi.org/10.1186/s13028-014-0065-z
39. Kozłowski J. Expansion of the invasive slug species *Arion lusitanicus*, Mabille, 1868 (Gastropoda: Pulmonata: Stylommatophora) and dangers to garden crops – a literature review with some new data. Folia Malacol. 2011;19(4):249–258. https://doi.org/10.2478/v10125-011-0005-8
40. Benson DA, Karsch-Mizrachi I, Clark K, Lipman DJ, Ostell J, Sayers EW. GenBank. Nucleic Acids Res. 2012;40:48–53. https://doi.org/10.1093/nar/gkr1202
41. Clough SJ, Bent AF. Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J. 1998;16(6):735–743. https://doi.org/10.1046/j.1365-313x.1998.00343.x
42. Bechtold N, Ellis J, Pelletier G. In planta *Agrobacterium*-mediated gene transfer by infiltration of adult *Arabidopsis thaliana* plants. Comptes Rendus de l'Académie des Sciences. 1993;316:1194–1199.
43. Miché L, Balandreau J. Effects of rice seed surface sterilization with hypochlorite on inoculated *Burkholderia vietnamiensis*. Appl Environ Microbiol. 2001;67(7):3046–3052. https://doi.org/10.1128/AEM.67.7.3046-3052.2001
44. Whelan RJ. An artificial medium for feeding choice experiments with slugs. J Appl Ecol. 1982;19:89–94. https://doi.org/10.2307/2402993
45. R Core Team. R: a language and environment for statistical computing. Vienna: R

Foundation for Statistical Computing; 2016.

46. Ercolani GL. Distribution of epiphytic bacteria on olive leaves and the influence of leaf age and sampling time. Microb Ecol. 1990;21(1):35–48. https://doi.org/10.1007/BF02539143
47. Piechulla B, Degenhardt J. The emerging importance of microbial volatile organic compounds. Plant Cell Environ. 2014;37:811–812. https://doi.org/10.1111/pce.12254
48. Matson PA, Parton WJ, Power AG, Swift MJ. Agricultural intensification and ecosystem properties. Science. 1997(277):504–509. https://doi.org/10.1126/science.277.5325.504
49. Zurek L, Ghosh A. Insects represent a link between food animal farms and the urban environment for antibiotic resistance traits. Appl Environ Microbiol. 2014;80(12):3562–3567. https://doi.org/10.1128/AEM.00600-14
50. Tucker CM, Fukami T. Environmental variability counteracts priority effects to facilitate species coexistence: evidence from nectar microbes. Proc Biol Sci. 2014;281(1778):20132637. https://doi.org/10.1098/rspb.2013.2637

Epifityczne bakterie na liściach sałaty modyfikują zachowania pokarmowe inwazyjnego ślimaka z rodziny ślinikowatych

Streszczenie

Ekologicznych interakcji roślin i zwierząt nie można rozpatrywać wyłącznie jako odizolowanych relacji dwóch gatunków, ich tłem bowiem są rozliczne zespoły drobnoustrojów. Współczesne badania wskazują, że poza bezpośrednim wpływem na gospodarza, mikroorganizmy związane z roślinami mogą modyfikować zachowanie organizmów z wyższych poziomów troficznych. Niestety, w kontekście roślinożerności ekologiczna funkcja niepatogennych bakterii kolonizujących liście pozostaje niezbadana. Nasze badania wykazały, że naturalnie występujące epifityczne bakterie wpływają na zachowania pokarmowe niewyspecjalizowanego roślinożercy. Aby to osiągnąć zastosowaliśmy epifityczne bakterie wyizolowane z liści *Lactuca sativa* var. *capitata* i określiliśmy ich wpływ na wybory pokarmowe ślinika *Arion vulgaris*. Sztuczne substraty pokarmowe oraz gnotobiotyczne liście sałaty zaszczepione koloniami bakteryjnymi zaoferowano ślimakom w różnych testach behawioralnych. Znaczący odsetek zastosowanych szczepów bakteryjnych spowodował zmianę trybu żerowania zwierząt eksperymentalnych. Odpowiedź ta mogła być dalej modyfikowana antybiotykami podawanymi zwierzętom przed testami behawioralnymi, co wskazuje, że zarówno bakterie występujące na roślinach jak i na zwierzętach mogą istotnie wpływać na interakcje roślina–zwierzę. Nasze badania wskazują na istotną rolę bakterii w modyfikowaniu relacji roślin i zwierząt oraz sugerują znaczący wpływ tych mikroorganizmów na zjawisko roślinożerności zarówno w systemach naturalnych, jak i w kontekście upraw ogrodniczych czy rolnych.