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Microbial symbioses : from vertebrates to invertebrates and back again

Panagiotis Sapountzis

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Université Clermont Auvergne

Mémoire présenté pour l'obtention de l'Habilitation à Diriger des Recherches

Spécialité : *écologie microbienne / microbiologie moléculaire*

***Microbial symbioses: from vertebrates to invertebrates and
back again***

Panagiotis (Panos) SAPOUNTZIS

Soutenance 7 Dec 2022

Membres du Jury

Sylvie DEQUIN	<i>Directrice de Recherche, INRAE Paris</i>	<i>Rapporteur</i>
Abdelaziz Heddi	<i>Professeur, Université INSA Lyon</i>	<i>Rapporteur</i>
Stéphane Chaillou	<i>Directeur de Recherche, INRAE Paris</i>	<i>Rapporteur</i>
Valérie Livrelli	<i>Professeur, Université Clermont Auvergne</i>	<i>Examineur</i>
Evelyne Forano	<i>Directeur de Recherche, INRAE Theix</i>	<i>Examineur</i>
Pierre Peyret	<i>Professeur, Université Clermont Auvergne</i>	<i>Tuteur</i>

UMR MEDIS, Centre Auvergne Rhône-Alpes, 63122 Saint-Genès-Champanelle

Remerciements

There are a lot of people I need to thank, I will try to remember everyone. A lot of people at MEDIS have helped me, but my very special thanks to Pierre, Mickael, Sabine and Evelyne. They have always been flexible enough to facilitate my demands when the bureaucratic cogs wouldn't turn (or were taking too long), or when I needed someone to mediate for finding a house or to vent when a grant got rejected, or the coffee machine was broken. Thank you.

A big thank you to my past mentors, colleagues and friends at Bourtzis lab, Bf2i and the centre for Social Evolution. Without you I would never be who I am today.

A very special thank you to my family: my parents, my siblings and my nephews. I miss you and I promise I will start visiting more often. You have always supported my professional decisions and encouraged me to believe in myself.

Last but not least, my better half, Alma. My favorite human, who makes my everyday great. Thank you not only for the joy you bring to my life, but also for encouraging me to stick to my guns and follow my dreams.

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Introduction

In recent decades, we have come to realize that bacterial endosymbionts have contributed to the functionality of most, if not all animal species (Kostas Bourtzis and Miller 2003; Dubilier, Bergin, and Lott 2008; Hara et al. 2013; Kamada et al. 2013; Kanji et al. 2017). Both vertebrates and invertebrates maintain numerous relationships with microbes that require specific genetic machinery to interact productively with their hosts, so that they are maximally likely to provide beneficial services and unlikely to become pathogenic.

I have always been fascinated by: i) the mechanisms governing host-bacteria and bacteria-bacteria interactions, ii) understanding the stability of these symbioses when they are mutually beneficial, and iii) the factors (e.g. host, diets, environment) that make microbes express harmful phenotypes that are costly for the animal host as they can constrain development, reduce nutrition, compromise health or even cause behavioral changes and increased aggression.

During my entire career, which started when I was an undergraduate and did my first internship at IMBB in 2001, I have been studying animal-bacterial symbioses in different systems. My training started with bacteria associated with model insects because insects are associated with a wide range of bacteria (Baumann et al. 1995; Kostas Bourtzis and Miller 2003; Douglas 1989; Moreira et al. 2009; Perrot-Minnot, Guo, and Werren 1996), can be easily reared in the laboratory and generation times are short. I then moved to the more complex symbiotic model systems of social insects, which can be used to make predictions as an analogy about human societies, and in my current positions I focus on the cattle intestinal microbiota. However, as I will explain in the last part of this thesis, I plan to use my expertise in insect bacterial symbioses to study relevant scientific questions in a novel insect model system.

CURRICULUM VITAE

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<https://scholar.google.dk/citations?user=AKD-YnsAAAAJ&hl=en>

EDUCATION

2007 - 2012, Training & courses: i) Training in protein isolation from polyacrylamide gels, for analysis by Mass Spectroscopy and in solution trypsin digestion and mass spec analysis using ESI-TRAP LC/MS/MS (in Spiros D. Garbis lab, in biomedical research foundation, academy of Athens, Greece), ii) Workshop on the preparation of scientific papers and abstracts in English entitled: “rédaction en anglais scientifique”, Lyon, France, iii) Bioinformatics workshop at University of Maryland, Institute for Genome Sciences, USA.

2003 - 2008 (awarded 2011), PhD: Department of Environmental and Natural Resources Management, University of Ioannina and Hellenic Pasteur Institute, Athens, Greece. Title: “Molecular characterization of the *Wolbachia* interactions with its hosts”. PhD defended in December 2008 and awarded in January 2011.

1998 - 2003, BSc: Department of Environmental and Natural Resources Management, University of Ioannina, Greece (5 year study). BSc thesis: Department of Biology, University of Wales, Bangor, UK.

2001, Laboratory internship during BSc: Institute of Molecular Biology and Biotechnology (IMBB), Heracleion, Crete, Greece, Prof. Babis Savakis lab.

POSITIONS

September 2019 – now: CRCN at the UMR 0454 MEDIS INRAE, Clermont-Ferrand, France.

June 2011 – May 2019: Senior postdoctoral researcher at the CSE, Department of Biology, University of Copenhagen, Denmark. From June 2012 – May 2014 funding was provided by a personal EU Marie-Curie Fellowship (IEF grant).

March 2009 – March 2011: Postdoctoral researcher – at the Functional Biology, Insects and Interactions (BF2I) lab, INRA, INSA-Lyon, France.

TEACHING

BSc courses:

2003-2006: Molecular Biology and Biochemistry (practical exercise sessions) University of Ioannina,

Greece (3 Academic years),

2003-2006: Microbiology (practical exercise sessions) University of Ioannina, Greece (3 Academic years),

MSc courses:

2011-2015: Genomic Approaches for Non-Model Organisms, University of Copenhagen, Denmark

2015-2016: Evolutionary Ecology Master course, University of Copenhagen, Denmark-

co-organizer of a mini symposium where the students had to read scientific articles and make presentations summarizing the results.

PhD course:

2016, 2018: Chemical and Genomic Insight to Host-Microbe Symbiotic Interactions, University of Copenhagen. 23-27 May 2016 & 13-18 May 2018 in Copenhagen, Denmark (program of lectures and practical exercises, in addition to teaching).

MEMBER OR EXAMINATION COMMITTEES

2016, Frida Carlsen, Project Outside Course Scope, 15 ECTS three month project, Oral exam 30min.

2016, Gustav B.Pedersen, Project Outside Course Scope, 15 ECTS three month project, Oral exam 30min.

2017, Ana Barrientos, Project Outside Course Scope, 15 ECTS three month project, Oral exam 30min.

2017, Kasun Bodawatta, Project Outside Course Scope, 15 ECTS three month project, Oral exam 30min.

RESEARCH GRANTS & AWARDS:

Participation as research assistant during my PhD:

2005-2006: Ministry of national education and religious affairs of Greece, EPEAEK II, Title: "Study of the ecological importance of the zooplankton community in Lake Trichonis, Greece".

2004-2006: Ministry of national education and religious affairs of Greece, Title: "Characterization of new ecotypes of *Arabidopsis thaliana* from Greece and molecular characterization of the *Arabidopsis* plant proteins that interact with bacterial effector proteins".

2006-2008: General secretariat for reasearch and technology, Title: "A biotechnological approach for the development of tomato plants that are resistant to bacterial speck disease".

2006-2008: General secretariat for reasearch and technology, Title: "Towards the development of a microarray system for the detection and characterization of plant pathogens".

Grants received/participated as PostDoctoral researcher and group leader:

2009-2011: Agence Nationale de la Recherche, France, Title: "Aphicibles". Participation as Research Assistant.

2012-2014: European Commission, IEF Marie-Curie Fellowship, FP7-PEOPLE-2011-IEF: "Gut Symbiomes of Fungus-growing Ants; GUTS", Grant number: 300584. Grant Recipient & Grant coordinator.

2013-2018: European Commission, ERC Advanced Grant, FP7-IDEAS-ERC: "Attine Ant Symbiomes (ANTS)", Grant agreement ID: 323085. Participation as Research Assistant.

2021-2023: Institute National de Recherche Agronomique et Environment, Metaprogrammes Holoflux. Title: "TANDEM". Participation as Collaborator and one of the three lead researchers responsible for the development of the bioinformatic analysis pipeline.

Grants awarded to students I supervised:

2015: An NSF Graduate Research Opportunities Worldwide (GROW) grant was awarded to PhD student Sarah J. Tolley in UCLA, whom I supervised at CSE (June 2015 to February 2016) and on the preparing of this grant proposal. Participation as Supervisor during project.

2018: A Smithsonian Institution Graduate Student Fellowship (travel grant) was awarded to Fulbright MSc student Veronica Sinotte at the University of Copenhagen. Host: Smithsonian Tropical Research Institute, Panama, May–August 2018. Participation as co-Supervisor during visit.

Travel Grants:

2002: ERASMUS travel grant to do part of my diploma thesis in Henk Braig's lab, Department of Biology, University of Wales, Bangor, UK. Grant Recipient.

2021: Institute National de Recherche Agronomique et Environment, Mission Scientifique a l'etranger; Travel grant to visit the Danish Technological University (Kgs Lyngby, Denmark) within the framework of an ongoing collaboration with Professor Frank Møller Aarestrup. Grant Recipient.

Awards:

2015: Cover photo of Applied Environmental Microbiology October Issue, <https://aem.asm.org/content/81/19.cover-expansion>

2018: Homepage cover photo on eLife November 2018 issue (not available online, preview from private record available here: <https://u.pcloud.link/publink/show?code=XZwxUWXZhqoll5bKDsjOoLFelB9f45oe0Pt7>)

OUTREACH ACTIVITIES:

I was involved in organizing and participating in Copenhagen Culture Night in 2014, a scientific exhibition event which had more than 20,000 visitors, and we exhibited ant colonies, explaining them and their symbionts to visitors.

(Sapountzis, Zhukova, et al. 2015) was highlighted in the American Society for Microbiology's official journal, *The Microbe* and has been presented (May 2016) in a science podcast (<http://www.bacteriofiles.com/2016/05/bacteriofiles-251-mycophagous.html>).

(Sapountzis, Gruntjes, et al. 2015) was highlighted by the editors as an article of significant interest (<https://aem.asm.org/content/81/19/6517>).

(Sapountzis et al. 2019) was highlighted by the editors in a perspective paper ([10.1111/mec.14998](https://doi.org/10.1111/mec.14998)).

(Sapountzis et al. 2018) has been presented in a separate digest <https://elifesciences.org/digests/39209/how-to-grow-a-fungal-garden>, has been highlighted as one of the most exciting latest research articles in the main homepage of the *eLife* journal and has also been

presented in <https://www.thenakedscientists.com/articles/interviews/fungus-farming-leaf-cutter-ants> by Chris Smith from the “Naked Scientists” (a BBC science podcast).

PROFESSIONAL SERVICES

Reviewer: I have reviewed articles for the Proceedings of the National Academy of Sciences of USA (PNAS), the ISME journal, Molecular Ecology, Frontiers in Microbiology (Review Editor), PLoS Pathogens, PLoS ONE, Scientific Reports, BMC Microbiology, Genomics, Environmental Microbiology, FEMS Microbiology letters, FEMS Microbiology Ecology, Insectes Sociaux and Science of Nature (previously named Naturwissenschaften), Microorganisms, Toxins and Life (MDPI) – (Also Topic Editor of Life), Animal Ecology, mBio and Environmental Entomology.

Scientific Membership: American Society for Microbiology.

ORGANIZATION OF SCIENTIFIC MEETINGS

2008 5th International *Wolbachia* Conference in Crete, Greece, 9-14 June 2008.

2021 12th International Symposium on Gut Microbiology (INRAE-Rowett) in Clermont Ferrand, France

PUBLICATION LIST

Twenty seven peer-reviewed publications (seven first and three last author) and an additional paper which is provisionally accepted and requires only minor edits (Zhukova et al. 2022). I am also the last author in a manuscript which is under review at Microbiome (Teseo et al.,) and three additional manuscripts in preparation, which will be finalized and submitted within 2022-23 (Ageorges et al. ; Sadowski et al. ; Teseo et al.). The six publications marked with an asterisk (*) were part of MSc/PhD thesis projects and student names (co-authorships) are presented in **bold and italics**.

h-index 22, i10-index 25, <https://scholar.google.com/citations?user=AKD-YnsAAAAJ&hl=en>

ResearchGate score: 28,44 <https://www.researchgate.net/profile/Panos-Sapountzis-2>

- 27 **Sapountzis P**, Teseo S, Otani S, Aarestrup FM, Forano E, Suen G, Tsiamis G, Haley B, Van Kessel JA, Huws SA. "FI: The Fecobiome Initiative." *Foodborne Pathogens and Disease*, December 21, 2021. <https://doi.org/10.1089/fpd.2021.0082>.
- 26 Bibbal D, Ruiz P, **Sapountzis P**, Mazuy-Cruchaudet C, Loukiadis E, Auvray F, Forano E, Brugère H. Persistent circulation of enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 in cattle farms: characterization of EHEC O157:H7 strains and fecal microbial communities of bovine shedders and non-shedders. *Frontiers in Veterinary Science* 2022 March doi.org/10.3389/fvets.2022.852475
- 25 **Sapountzis P**, Segura A, Desvaux M, Forano E. An Overview of the Elusive Passenger in the Gastrointestinal Tract of Cattle: The Shiga Toxin Producing *Escherichia coli*. *Microorganisms* 2020 8 (6), 877. doi: 10.3390/microorganisms8060877.
- 24 Sinotte VM, Renelies-Hamilton J, Taylor BA, Ellegaard KM, **Sapountzis P**, Vasseur Cognet M, Poulsen M. Synergies between division of labor and gut microbiomes of social insects. *Front. Microbiol.* 2020 Jan doi.org/10.3389/fevo.2019.00503
- 23 * **Tolley SJ**, Nonacs, P. and **Sapountzis P**. Wolbachia Horizontal Transmission Events in Ants: What Do We Know and What Can We Learn? *Front. Microbiol.* 7. Available from: <https://www.frontiersin.org/articles/10.3389/fmicb.2019.00296/full>. IF=4.08
22. Teseo S, vanZweden J, Pontieri L, Kooij PW, Sørensen SJ, Wenseleers T, Boomsma JJ, Poulsen M, **Sapountzis P**. The scent of symbiosis: gut bacteria affect social interactions in leafcutting ants. *Animal Behaviour*. 2019 Apr, 150: 239-254. IF=3.07
21. Otani S, Zhukova M, Koné NG, da Costa RR, Mikaelyan A, **Sapountzis P**, Poulsen M. Gut microbial compositions mirror division of labour in a major lineage of eusocial insects. *Environ. Microbiol. Reports*. 2018 Dec 16. doi: 10.1111/1758-2229.12728. IF=2.885
20. **Sapountzis P**, Zhukova M, Shik J, Schiøtt M, Boomsma JJ. (2018) Reconstructing the functions of endosymbiotic Mollicutes bacteria in fungus-growing ants. *eLIFE*. 2018 Nov 20;7. pii: e39209. doi: 10.7554/eLife.39209. (Cover photo on eLIFE homepage, highlighted in <https://elifesciences.org/digests/39209/how-to-grow-a-fungal-garden>, and featured in elife podcasts). IF=7.72

19. **Sapountzis P**, Nash DR, Schiøtt M, Boomsma JJ. (2018) The evolution of gut-microbiomes in fungus-growing ants. *Mol. Ecol.* 2018 Nov 9. doi: 10.1111/mec.14931. (highlighted in a perspective paper). IF=6.49
18. Zhukova M, **Sapountzis P**, Schiøtt M, Boomsma JJ. (2017). Diversity and Transmission of Gut Bacteria in *Atta* and *Acromyrmex* Leaf-Cutting Ants during Development. *Front. Microbiol.* 10. Available from: <https://www.frontiersin.org/articles/10.3389/fmicb.2017.01942/full>. IF=4.16
17. * **da Costa RR**, Hu H, Pilgaard B, Vreeburg S, Schückel J, Sydow Krogh Pedersen K, Krešimir Kračun S, Kamp Busk P, Harholt J, **Sapountzis P**, Lange L, Aanen D, Poulsen M. (2017). Enzyme activities at different stages of plant biomass decomposition in three species of fungus-growing termites. *Appl. Environ. Microbiol.* doi: 10.1128/AEM.01815-17.
16. * **Sapountzis P**, **de Verges J**, Rousk K, Cilliers M, Vorster BJ, Poulsen M. (2016). Potential for Nitrogen Fixation in the Fungus-Growing Termite Symbiosis. *Front. Microbiol.* 7. Available from: <http://journal.frontiersin.org/article/10.3389/fmicb.2016.01993/full>. IF=4.1615. **Sapountzis, P**, Zhukova, M., Hansen, L.H., Sørensen, S.J., Schiøtt, M., and Boomsma, J.J. (2015). *Acromyrmex* Leaf-Cutting Ants Have Simple Gut Microbiota with Nitrogen-Fixing Potential. **Appl. Environ. Microbiol.** 81, 5527–5537. (highlighted in *Microbe*, July 2015).
14. * **Liberti, J**, **Sapountzis, P**, Hansen, L.H., Sørensen, S.J., Adams, R.M.M., and Boomsma, J.J. (2015). Bacterial symbiont sharing in *Megalomyrmex* social parasites and their fungus-growing ant hosts. **Mol. Ecol.** 24, 3151–3169. IF=6.49, IF=6.49
13. * **Sapountzis, P**, **Gruntjes, T**, Otani, S., Estevez, J., da Costa, R.R., Plunkett, G., Perna, N.T., and Poulsen, M. (2015). The Enterobacterium *Trabulsiella odontotermitis* presents Novel Adaptations Related to Its Association with Fungus-Growing Termites. **Appl. Environ. Microbiol.** 81, 6577–6588. (Editor's choice and Issue cover photo).
12. **Sapountzis, P**, Duport, G., Balmand, S., Gaget, K., Jaubert-Possamai, S., Febvay, G., Charles, H., Rahbé, Y., Colella, S., and Calevro, F. (2014). New insight into the RNA interference response against *cathepsin-L* gene in the pea aphid, *Acyrtosiphon pisum*: molting or gut phenotypes specifically induced by injection or feeding treatments. **Insect Biochem. Mol. Biol.** 51, 20–32. IF=3.45
11. Rabatel, A., Febvay, G., Gaget, K., Duport, G., Baa-Puyoulet, P., **Sapountzis, P**, Bendridi, N., Rey, M., Rahbé, Y., Charles, H., et al. (2013). Tyrosine pathway regulation is host-mediated in the pea aphid symbiosis during late embryonic and early larval development. **BMC Genomics** 14, 235. IF=3.99
10. * Um, S., **Fraimout, A**, **Sapountzis, P**, Oh, D.-C., and Poulsen, M. (2013). The fungus-growing termite *Macrotermes natalensis* harbors bacillaene-producing *Bacillus* sp. that inhibit potentially antagonistic fungi. **Sci. Rep.** 3, 3250. IF=5.58
9. Andersen, S.B., Hansen, L.H., **Sapountzis, P**, Sørensen, S.J., and Boomsma, J.J. (2013). Specificity and stability of the *Acromyrmex-Pseudonocardia* symbiosis. **Mol. Ecol.** 22, 4307–4321. IF=6.49
8. Poulsen, M., and **Sapountzis, P**. (2012). Behind every great ant, there is a great gut. **Mol. Ecol.** 21, 2054–2057. IF=6.49

7. Saridaki, A., **Sapountzis, P.**, Harris, H.L., Batista, P.D., Biliske, J.A., Pavlikaki, H., Oehler, S., Savakis, C., Braig, H.R., and Bourtzis, K. (2011). *Wolbachia* prophage DNA adenine methyltransferase genes in different *Drosophila-Wolbachia* associations. **PLoS ONE** 6, e19708. IF=3.23
6. Bordenstein, S.R., Paraskevopoulos, C., Dunning Hotopp, J.C., **Sapountzis, P.**, Lo, N., Bandi, C., Tettelin, H., Werren, J. H., and Bourtzis, K. (2009). Parasitism and mutualism in *Wolbachia*: what the phylogenomic trees can and cannot say. **Mol. Biol. Evol.** 26, 231–241. IF=9.11
5. Cáceres, C., Segura, D.F., Vera, M.T., Wornoayporn, V., Cladera, J.L., Teal, P., **Sapountzis, P.**, Bourtzis, K., Zacharopoulou, A., and Robinson, A.S. (2009). Incipient speciation revealed in *Anastrepha fraterculus* (Diptera; Tephritidae) by studies on mating compatibility, sex pheromones, hybridization, and cytology. **Biol. J. Linnean Soc.** 97, 152–165. IF=2.26
4. Klasson, L., Westberg, J., **Sapountzis, P.**, Näslund, K., Lutnaes, Y., Darby, A.C., Veneti, Z., Chen, L., Braig, H.R., Garrett, R., Bourtzis, K., and Andersson, S.G. (2009). The mosaic genome structure of the *Wolbachia* wRi strain infecting *Drosophila simulans*. **Proc. Natl. Acad. Sci. U.S.A.** 106, 5725–5730. IF=9.67
3. Kounatidis, I., Crotti, E., **Sapountzis, P.**, Sacchi, L., Rizzi, A., Chouaia, B., Bandi, C., Alma, A., Daffonchio, D., Mavragani-Tsipidou, P., and Bourtzis, K. (2009). *Acetobacter tropicalis* is a major symbiont of the olive fruit fly (*Bactrocera oleae*). **Appl. Environ. Microbiol.** 75, 3281–3288. IF=3.67
2. Siozios, S., **Sapountzis, P.**, Ioannidis, P., and Bourtzis, K. (2008). *Wolbachia* symbiosis and insect immune response. **Insect Science** 15, 89–100. IF=2.14
1. Ioannidis, P., Dunning Hotopp, J.C., **Sapountzis, P.**, Siozios, S., Tsiamis, G., Bordenstein, S.R., Baldo, L., Werren, J.H., and Bourtzis, K. (2007). New criteria for selecting the origin of DNA replication in *Wolbachia* and closely related bacteria. **BMC Genomics** 8, 182. IF=3.99

INTERNATIONAL CONFERENCES AND INVITED SEMINARS

- I have given two **invited seminars**, one at the Hellenic Pasteur Institute on my PhD work, and one at the University of Copenhagen on my work on gut bacteria of fungus-growing ants. I was also invited to give a talk at the 18th quadrennial World Congress of the International Society for the Study of Social Insects (IUSSI) in Brazil (5-10 August 2018) on my work on Mollicutes gut bacteria but unfortunately there was no budget for my trip and stay.

TALKS

18. **P. Sapountzis**, M. Zhukova, JZ Shik, DR Nash, M. Schiott, JJ. Boomsma. Did the success of leaf-cutting ants depend on the sequential domestication of Mollicutes endosymbionts? Royal Entomological Society meeting on Endosymbiont Infection and Immunity, Liverpool, UK, 21-22 March 2018.
17. M. Zhukova, **P. Sapountzis**, M. Schiott, JJ. Boomsma. Gut bacterial transmission strategies in leaf-cutting ants. 6th European Congress of the International Union for the Study of Social Insects (IUSSI), Helsinki, Finland, 8-11 August 2016.

16. T. Innocent, N. Holmes, M. Schiøtt, **P. Sapountzis**, D. Yu, M. Hutchings, JJ. Boomsma. The maintenance of defensive microbiomes and the avoidance of antibiotic resistance. 6th European Congress of the International Union for the Study of Social Insects (IUSSI), Helsinki, Finland, 8-11 August 2016.
15. J. de Verges, **P. Sapountzis**, K. Rousk, M. Du Plessis, B.J. Vorster and M. Poulsen. Do nitrogen-fixing bacteria help fungus-growing termites overcome the nitrogen limitations of plant-based farming? (poster and flash talk). Annual Symposium of the Copenhagen Microbiology Centre, Denmark, 2 October 2015.
14. J. de Verges, **P. Sapountzis**, and M. Poulsen. Do nitrogen-fixing bacteria help fungus-growing termites overcome the limitations of plant-based farming? Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa, 23 January 2015.
13. J. de Verges, **P. Sapountzis**. Do nitrogen-fixing bacteria help fungus-growing termites overcome the nitrogen limitations of plant-based farming? (poster and flash talk). Annual Symposium of the Copenhagen Microbiology Center, Denmark, 3 October 2014.
12. J. Liberti, **P. Sapountzis**, LH. Hansen, SJ. Sørensen, RMM. Adams, JJ. Boomsma. Microbiomes of *Megalomyrmex* social parasites and their fungus-growing ant hosts. 17th World Congress of the International Union for the Study of Social Insects (IUSSI), Cairns, Australia, 13-18 July 2014.
11. **P. Sapountzis**, L. H. Hansen, S. J. Sørensen, M. Schiøtt and J. J. Boomsma. Phylogenetic distribution, stability and function of gut bacterial endosymbionts in fungus-growing ants. 17th World Congress of the International Union for the Study of Social Insects (IUSSI), Cairns, Australia, 13-18 July 2014.
10. **P. Sapountzis**, L. H. Hansen, S. J. Sørensen, M. Schiøtt and J. J. Boomsma. Phylogenetic distribution, stability and function of gut bacterial endosymbionts in fungus-growing ants. Invited seminar for the Section for Ecology and Evolution at the Biology Department, University of Copenhagen, Denmark, 2013.
9. **P. Sapountzis**, L. H. Hansen, S. J. Sørensen, M. Schiøtt and J. J. Boomsma. Mapping the gut bacteria of fungus-growing ants. Annual Meeting of the NW European Section or IUSSI, London, UK, 10 December 2012.
8. M. Poulsen, **P. Sapountzis**, J. Liberti, S. Otani, R. M. M. Adams, M Schiøtt, J J. Boomsma. Exploring the roles of microbiomes in the guts of fungus-farming social insects. Annual Symposium of the Copenhagen Microbiology Center, Copenhagen, Denmark, October 5, 2012.
7. **P. Sapountzis**, G. Duport, K. Gaget, S. Balmand, S. Jaubert-Possamai, G. Febvay , H. Charles, Y. Rahbé, S. Colella et F. Calevro. *Effet de la méthode d'administration (ingestion versus injection) du traitement RNAi chez Acyrthosiphon pisum*. Réseau Francais de la Biologie Adaptive du Puceron. 3rd meeting Lyon, France, 20-21 October 2010 (talk in French).
6. **P. Sapountzis**, H. Boleti, S. R. Bordenstein, K. Bourtzis. Insect-*Wolbachia*-WO phage tripartite symbiotic interaction: exploring the role of phage. 23rd International Congress of Entomology, International Convention Centre Durban, South Africa, 6-12 July 2008.
5. **P. Sapountzis**, S. Siozios, P. Ioannidis, A. Saridaki, H. Boleti, K. Bourtzis *Drosophila-Wolbachia* symbiotic interactions: exploring the role of ankyrins. 23rd International Congress of Entomology, International Convention Centre Durban, South Africa, 6-12 July 2008.

4. S. R. Bordenstein, C Paraskevopoulos, J. C. Dunning Hotopp, **P. Sapountzis**, N. Lo, C. Bandi, H. Tettelin, J. H. Werren and K. Bourtzis. Phylogenomics of *Wolbachia*: what the tree can and cannot say. 5th International *Wolbachia* Conference. Kolymbari Crete, Greece, 9-14 June 2008.
3. **P. Sapountzis**, K. Moraiti, I. Kounatidis, S. Siozios, P. Mavragani-Tsipidou, Nikos T. Papadopoulos and K. Bourtzis. *Wolbachia* symbiosis in Tephritidae. Fruit flies's microflora – 1st Meeting of Tephritid workers of Europe And Middle east (TEAM). Palma of Mallorca. 7-8 April 2008.
2. **P. Sapountzis**. Molecular characterization of the *Wolbachia* interactions with it's hosts. Invited talk at the Hellenic Pasteur Institute, Greece, January 2009.
1. P. Ioannidis, J. C. Dunning Hotopp, **P. Sapountzis**, S. Siozios, G. Tsiamis, S.R. Bordenstein, L. Baldo, J.H. Werren, and K. Bourtzis. On the Origin of DNA Replication of *Wolbachia* and Closely Related Bacteria. 4th International *Wolbachia* Conference. Puerto Rico, 24-29 June 2006.

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23. V. A Sadowski, P. Sapountzis, P Kooij, JJ. Boomsma, RMMA, Adams. Vertical transmission of bacteria by farmer ants. ESA, ESC and ESBC 13-16 Nov 2022, Vancouver, Canada.
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21. **P. Sapountzis**, M. Zhukova, A. S. Fomsgaard, DR. Nash, M. Schiott, JJ. Boomsma. Mollicutes bacteria: new mutualistic endosymbionts in fungus-growing ant guts? 6th European Congress of the International Union for the Study of Social Insects (IUSSI), Helsinki, Finland, 8-11 August 2016.
20. **P. Sapountzis**, M. Zhukova, Hansen LH, Sørensen SJ, M. Schiott, JJ. Boomsma. The simplicity and complexity of the attine ant gut microbiota: living within a symbiotic network. 15th Congress of the European Society for Evolutionary Biology (ESEB), Lausanne, Switzerland, 10-14 August 2015.
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18. J. Liberti, **P. Sapountzis**, LH. Hansen, SJ. Sørensen, RMM Adams, JJ. Boomsma. Specificity and transmission of bacterial endosymbionts in *Megalomyrmex* social parasites and their fungus-growing ant hosts. 14th Congress of the European Society for Evolutionary Biology (ESEB), Lisbon, Portugal, 19-24 August 2013.
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14. **P. Sapountzis**, G. Duport, K. Gaget, S. Balmand, S. Jaubert-Possamai, G. Febvay , H. Charles, Y. Rahbé, S. Colella et F. Calevro. Réponse tissu-spécifique au traitement par RNAi chez le puceron du pois : une étude cinétique à l'échelle de l'individu. 16ème Colloque de biologie de l'Insecte (CBI), Lyon France, 18-20 October 2010.
13. **P. Sapountzis**, G. Duport, K. Gaget, T. Horn, M. Boutros, G. Febvay, S. Jaubert-Possamai, H. Charles, Y. Rahbé, S. Colella, and F. Calevro. Single insect and tissue compartment specific response to RNAi microinjection in aphids: each individual matters. 4th Annual Arthropod Genomics Symposium, Kansas City, USA, 10-13 June 2010.
12. A. Saridaki, **P. Sapountzis**, S. Siozios, P. Ioannidis, H. Boleti, Sofia Zabalou, Charalambos Savakis and K. Bourtzis. *Wolbachia* safe sex: The triggering factor for speciation, ecological diversity and applications. 1st Mikrobiokosmos (MBK) Conference, Athens, Greece, 12-14 December, 2008.
11. A. Saridaki, **P. Sapountzis**, S. Siozios, P. Ioannidis, H. Boleti, and K. Bourtzis. *Wolbachia* ankyrins and their potential role on *Drosophila*-*Wolbachia* symbiosis. FEBS Journal 275:266.
10. S. Siozios, **P. Sapountzis**, P. Ioannidis, A. Saridaki, and K. Bourtzis. *Drosophila*-*Wolbachia* symbiotic associations: exploring the role of wMel ankyrins. 5th International *Wolbachia* Conference, Kolymbari-Crete, Greece, 9-14 June, 2008.
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8. **P. Sapountzis**, H. Boleti, SR. Bordenstein and K. Bourtzis. A novel tool to study insect-*Wolbachia* phage interactions. 5th International *Wolbachia* Conference, Kolymbari-Crete, Greece, 9-14 June, 2008.
7. I. Kounatidis , E. Crotti, **P. Sapountzis**, Luciano Sacchi, Bessem Chouaia, Claudio Bandi, Alberto Alma, Daniele Daffonchio, P. Mavragani-Tsipidou and K. Bourtzis. What bacterial species constitute the symbiotic flora of the olive fly, *Bactrocera oleae*? Fruit flies' microflora. 1st Meeting of TEAM. Palma of Mallorca. 7-8th April. 2008.
6. I. Kounatidis , E. Crotti, **P. Sapountzis**, Luciano Sacchi, Claudio Bandi, Daniele Daffonchio, P. Mavragani-Tsipidou and K. Bourtzis. The symbiotic flora of a lab strain of the olive fly, *Bactrocera oleae*. 12th Hellenic Conference of Entomology, Larnaka, Cyprus, 13-16 November, 2007.5. K. Moraiti, I. Kounatidis, **P. Sapountzis**, P. Mavragani-Tsipidou, N. T. Papadopoulos and K. Bourtzis. *Wolbachia* in tephritidae. 12th Hellenic conference of entomology, Larnaka, Cyprus, 13-16 November, 2007.
4. **P. Sapountzis**, D. A. Voronin, S. Siozios, L. Sacchi, C. Bandi, E. Kiseleva, C. Savakis and K. Bourtzis. Characterization of the symbiotic relationship of *Wolbachia* and it's host using TEM and confocal microscopy. 12th Hellenic conference of entomology, Larnaka, Cyprus, 13-16 November, 2007.

3. S. Siozios, **P. Sapountzis**, P. Ioannidis, G. Tsiamis and K. Bourtzis. Distribution and expression study of the bacterial ankyrins in the symbiotic relationship *Drosophila-Wolbachia*. Meeting of the Hellenic Society for Bioscientists. "Biosciences in the 21st century", Athens, Greece, 13-15 April, 2006.
2. P. Ioannidis, J. C. Dunning Hotopp, **P. Sapountzis**, S. Siozios, G. Tsiamis, S.R. Bordenstein, L. Baldo, J.H. Werren, and K. Bourtzis. On the Origin of DNA Replication of *Wolbachia* and Closely Related Bacteria. Meeting of the Hellenic Society for Bioscientists. "Biosciences in the 21st century", Athens, Greece, 13-15 April, 2006.
1. S. Siozios, **P. Sapountzis**, P. Ioannidis, L. Klasson, A. Darby, K. Näslund, H. R. Braig, S. G. E. Andersson and K. Bourtzis. Distribution and expression analysis of bacterial ankyrin genes in different *Drosophila-Wolbachia* symbiotic associations. 4th International *Wolbachia* Conference, Puerto Rico, 24-29 June, 2006.

SUPERVISION

PhD students:

2015-2018: Sarah J. Tolley (co-supervised, visiting PhD student from UCLA, USA), PhD title: Evolutionary Effects of a Eusocial Host Life History on an Endosymbiont. 50% co-supervision during 2015-2017.

2014-2017: Rafael da Costa (co-supervised, PhD student at UCPH), PhD title: The Physiology of Microbial Symbionts in Fungus-Farming Termites. 20% co-supervision during 2015-2017.

MSc students:

2013: Antoine Fraimout (Biological Research project, co-supervised at UCPH), A genomic approach to deducing the role of *Bacillus* sp. in *Macrotermes natalensis*, 50% co-supervision

2014: Joanito Liberti (MSc thesis, co-supervised at UCPH), *Megalomyrmex* and attine host ant microbiomes, symbiont sharing of their gut microbes. 33% co-supervision

2015: Jane de Verges (MSc thesis, co-supervised at UCPH), Do nitrogen-fixing bacteria help fungus-growing termites overcome the nitrogen limitations of plant-based farming? 50% co-supervision

BSc students (after 2006):

2006: Antonis Nithaurianakis (BSc thesis, co-supervised at UOI, Greece), lab supervision in molecular and microbiological techniques 50% co-supervision

2008: Maria Kotini (BSc thesis, co-supervised at Hellenic Pasteur Institute, Greece currently UCL, UK), lab supervision in molecular and cell biology techniques 50% co-supervision

2010: Francois Cochet, IUT stagiaire (co-supervised at Bf2i, France), lab supervisor in molecular biology (RNA extraction and qRT-PCR) techniques

2014: Oluf Sonne Georg (BSc thesis, supervised at UCPH), establishment of the RNAi technique in ants

2014: Thijs Gruntjes (ERASMUS project, co-supervised, 2014 at UCPH), *Trabulsiella odontotermis*, a new bacterial symbiont found in fungus-growing termite guts, 50% co-supervision

2015: Anna Fomsgaard (fagprojekt, supervised, 2015 at UCPH), symbiotic bacteria in FG ants

2016: Anna Fomsgaard (Bachelor thesis, supervised at UCPH), Mollicutes bacteria in *Acromyrmex echinator* ants

2021: Alice Leboucher, IUT stagiaire (supervised at INRAE, Clermont-ferrand centre, April 2021-June 2021), "Caractérisation moléculaire de souches bactériennes isolées de fèces bovines".

2021: Lucie Levet, IUT stagiaire (supervised at INRAE, Clermont-ferrand centre, April 2022-June 2022), "Caractérisation moléculaire de différents pathogènes présents dans des échantillons fécaux de bovin".

Below I present my scientific career chronologically. I start with my introduction to research, as an undergraduate student in 2001, and then I present my diploma thesis, which was a continuation of a summer project in 2001. Then, follows my PhD thesis, which took place at the University of Ioannina and the Pasteur Institute in Greece. After finishing my PhD, I moved -permanently- abroad for my postdoctoral period first in France and then in Denmark. Finally, I finish up with my current work as CRCN at MEDIS and I lay down some ideas about future projects.

Sandwich course at Heracleion, Crete, Institute of Molecular Biology and Biotechnology (2001)

My undergraduate degree at the University of Ioannina, Greece (UOI) provided me with my first experience in the research field of insects, bacterial symbionts and molecular biology. My first acquaintance with a molecular biology lab was during a Sandwich course at the Institute of Molecular Biology and Biotechnology (IMBB) at Heracleion Crete in Greece organized by Prof. Babis Savakis from July till August 2001. During this course I used molecular approaches to characterize the microbiota associated with *Bactrocera olea*, a major agricultural pest in southern Europe and possibly the worst pest for Greek agriculture. Traditional methods used for pest control include chemical insecticides, which have been tried for the greater part of a century, but their non specificity, wide target range and long half-life in the environment have rendered them unsuitable for environmental sustainability and an eco-friendly 'green' growth. One of the most promising approaches that has been proposed is the use of naturally occurring endosymbionts, which may be able to reduce the insect pests reproduction rates or prevent them from attacking agricultural crops of economical significance. For such approaches to work, we would need to identify bacterial symbionts that can fast and easily transmit across the focal insect species and/or are able to induce phenotypes that can stall reproduction rates, induce mortality or other deleterious effects or prevent the insect host from attacking agricultural crops with the use of pheromones or the provision of a more complete diet (e.g. Amino acids, vitamins, which could potentially make the insect host alter its nutritional niche). During this project, I focused on characterizing bacterial symbionts of *Bactrocera olea*. To this end, I used 16S targeted PCR to amplify and clone part of the 16S gene from different insect individuals, in order to map the frequency and distribution of the identified symbionts. The amplified 16S PCR products were ligated and cloned in commercially available vectors and successfully cloned 16S gene fragments grew on agar plates with antibiotic selection. After confirming that the correct 16S fragment had been cloned (PCR and electrophoresis) I reamplified the 16S fragments to perform a Restriction Fragment Length Polymorphism analysis. Representatives from each group of different 16S variants were sent for Sanger sequencing.

Diploma thesis (2002-2003)

During my diploma thesis work, I continued working with endosymbiotic bacteria of insects, but I was introduced into a new model system; the *Drosophila Wolbachia* symbiotic relationship. *Wolbachia* bacteria hold great promise as novel and environmentally friendly bio-control agents as they are able to induce a variety of reproductive abnormalities in insect host, such as cytoplasmic incompatibility, parthenogenesis, feminization and killing of males. Cytoplasmic incompatibility is the most common reproductive abnormality and it essentially prevents populations of the same insect species to produce viable offsprings unless they carry the same *Wolbachia* strains; hence making the populations incompatible. *Wolbachia* bacteria are able to achieve that by targeting cell cycle regulatory proteins causing asynchrony between male and female pronuclei (see also below). Hence *Wolbachia* strains that are able to induce these modifications are known as “*mod+*”. If the same *Wolbachia* strain is present in the female reproductive organs, this asynchrony is restored and normal embryos are produced (Tram and Sullivan 2002), and thus the strains are able to “rescue” the offsprings and are “*resc+*”.

The potential applications from the exploitation of these phenotypes are great if we consider that *Wolbachia* is found in possibly up to 75% of all insects, the most populous group (class) of animals which includes many species of environmental, economic and medical importance. Therefore, research related to *Wolbachia* bacteria is mainly divided in three parts: i) the identification and characterization of *Wolbachia* infections in insect hosts, ii) the deciphering of molecular and cellular machinery that *Wolbachia* utilizes to exploit their host and induce phenotypes and iii) practical applications for insect biocontrol. My diploma thesis consisted of two parts; the first part was the identification of molecular components responsible for the communication/interaction of *Wolbachia* cells with insect host cells and the second part was the identification and characterization of novel *Wolbachia* strains in insect hosts.

The object of the first part of my work was the identification of proteins that are responsible for the communication of *Wolbachia* with insect host cells and the lab work for this part took place in the Department of Biology, University of Wales, Bangor, UK in Prof. Henk Braig's lab. We hypothesized that the proteins of interest would be present in the outer membrane that surrounds *Wolbachia* bacteria and very likely comes from the host (host vacuole). Some of these putative membrane proteins are believed to play a role in feeding *Wolbachia*, suppressing the host's immune system (and therefore prevent any host immune responses) and/or facilitates the vertical transmission of *Wolbachia* to the offsprings as *Wolbachia* bacteria are known to be able to interact with the cytoskeleton of their insect host in order to survive, proliferate and exploit the host reproduction. During this part of my thesis, I and Panos Ioannidis, a classmate who participated in this project, developed a platform which made feasible to extract, isolate and purify outer membrane *Wolbachia* proteins from reproductive tissues of *Drosophila* flies and characterize their properties using 2D gels. The advantage of using 2D gels is that the characterized proteins can be subsequently (and relatively easy) isolated and sent for mass spectroscopy sequencing, even though it was, unfortunately, not possible within the timeframe of my diploma thesis.

In addition to the above research, which was conducted using strains of *Drosophila simulans*, two other species of insects were studied to determine if they were infected with *Wolbachia*. One of them is *Bactrocera oleae*, which was also the focus of my sandwich course in IMBB and the other the parasitic *Chrysopa sp.* In

the context of my diploma thesis, the endosymbiotic flora of individuals of the insect *Bactrocera oleae* was studied by PCR. Populations from different parts of Greece were used. Using 16S PCR and Sanger sequencing I confirmed the existence of symbiotic microbiota in almost all individuals of *Bactrocera oleae*, mainly composed of *Pseudomonas* bacteria. It is worth noting that the *Wolbachia* bacterium was absent from all *Bactrocera oleae* populations studied, but *Chrysopa* sp, was found to be infected with *Wolbachia*. *Chrysopa* sp. are carnivorous parasites, known as green lace wings, and can grow rapidly in greenhouses and the open field. Adults have a light green, transparent wings in which the veins are visible (hence the name lacewings). The characterization of this insect as carnivorous is not based on the characteristics of adults as they feed on nectar, honeydew, and pollen. In contrast, the larvae of this insect are very greedy consuming a variety of different organisms, especially when they are in the third stage. This feature makes them effective in biological control methods of many crop pests.

PhD thesis (2003-2008; officially awarded 2011)

After my graduation, I decided to continue in the same field and started a PhD in Associate Professor Kostas Bourtzis lab. Following the research that I started during my diploma thesis, I continued working on the identification of molecular components responsible for the communication/interaction of *Wolbachia* cells with host cells and the identification and characterization of novel *Wolbachia* strains in various insect hosts (Sapountzis 2011).

Various *in silico* and wet lab approaches were undertaken to study surface proteins, ankyrins, the type IV secretion system (T4SS), and prophage elements of *Wolbachia*, all components that were either suspected or have demonstrated (in close relatives of *Wolbachia* bacteria such as *Anaplasma* and *Ehrlichia* species) to facilitate the communication and/or cause pathogenicity in their hosts. To study these components the work was divided in two parts. The first part took place at Kostas Bourtzis lab in the Department of Environmental Management, UOI from September 2003 till September 2007, while the second part took place in the lab of molecular Parasitology in the Hellenic Pasteur Institute in Athens, under the supervision of Dr. Haralabia Boleti. During the first part of my PhD, I mostly worked on molecular biology and biochemistry techniques, while under the supervision of Dr. Boleti I was trained in cellular biology methods and fluorescent microscopy.

***Wolbachia* T4SS**

The presence of T4SS in *Wolbachia* was first described in two strains (*wTai* and *wKueYO*) that induce CI (Masui, Sasaki, and Ishikawa 2000). As part of my PhD, I identified the genes that constitute a functional T4SS in several strains: *virB8*, *virB9*, *virB10*, *virB11* and *virD4* (Masui, Sasaki, and Ishikawa 2000). The sequencing of the genome of various *Wolbachia* strains (*wMel*, *wRi*, *wBm*, *wPip* and *wVul*) showed that the T4SS of the bacterium *Wolbachia* is organized in different regions of the bacterial chromosome in the form of operons (Wu et al. 2004; Foster et al. 2005; Klasson et al. 2008; 2009; Félix et al. 2008). In the case of the *wMel* strain, one group of genes (*virB8*, *virB9*, *virB10*, *virB11* and *virD4*) is organized together with the *wspB* gene, (a paralog of the *wsp* gene), which encodes the major surface protein of the bacterium *Wolbachia* (Braig et al. 1998; Wu et al. 2004). I examined the expression of all T4SS genes using targeted primer pairs in three different symbiotic *Drosophila-Wolbachia* relationships. The three symbiotic relationships studied were: (a) *Drosophila simulans* (*wRi*) where high levels of CI expression have been observed (Ary A. Hoffmann, Turelli, and Simmons 1986) (b) *Drosophila melanogaster* (*wMel*) where lower levels of CI expression have been observed (A. A. Hoffmann and Turelli 1988; K. Bourtzis et al. 1994; 1996) and (c) *Drosophila simulans* (*wAu*) where no CI expression has been observed (Ary A. Hoffmann, Turelli, and Simmons 1986). Based on the mod / resc model, *wRi* and *wMel* are considered mod+ resc+, while *wAu* is considered a mod- resc strain (Werren 1997; D. Poinot et al. 1998; Zabalou et al. 2008). I further examined the expression of T4SS genes at different developmental stages and tissues; in embryos, testes, and ovaries, as well as in adult males and females in order to determine whether there is a correlation between the expression levels and the induction of the CI phenotype.

Previous research in intracellular bacteria has shown that T4SS plays an important role in infectivity, survival and reproduction in host cells (Christie and Vogel 2000; Sexton and Vogel 2002; Cascales and Christie 2003). For this reason, T4SS is thought to play an important role in the biology of *Wolbachia*. The results of this chapter of my PhD show that almost all structural components of the T4SS are expressed in almost all

the developmental stages and tissues of the three strains studied (*wMel*, *wRi* and *wAu*). It should be mentioned that the genes coding for the structural components of the T4SS have a conserved structure both in *Wolbachia* genomes as well as in those of bacterial strains related to *Wolbachia* such as *Ehrlichia canis*, *Ehrlichia ruminantium*, *Anaplasma phagocytophila*, *Ehrlichia chaffeensis*, *Rickettsia sibirica*, and *Anaplasma marginale* (Masui, Sasaki, and Ishikawa 2000; Brayton et al. 2005; Collins et al. 2005; Malek et al. 2004; Wu et al. 2004; Foster et al. 2005; Mavromatis et al. 2006; Klasson et al. 2008; 2009; Félix et al. 2008). Specifically, they are organized into two groups of genes that are at different positions in the bacterial genome. I confirmed the expression of the *virB8*, *virB9*, *virB10*, *virB11*, *virD4* genes and that they are part of the same operon. Another important observation was the absence of genes coding the *VirB2* and *VirB5* proteins. These proteins are necessary for the formation of the pilus which usually activates T4SS, however these structural components in *Wolbachia*, which is strictly intracellular, do not seem necessary (Félix et al. 2008), as the presence of the pilus may have been rendered obsolete over evolutionary time.

Investigation of *Wolbachia* surface proteins

Anaplasma marginale is an intracellular pathogenic bacterium, phylogenetically related to *Wolbachia*. An important role in the infectivity of this bacterium is played by its major surface protein 1a (MSP1a). MSP1a has been shown to assist *Anaplasma* in attaching itself to bovine erythrocytes (J. de la Fuente et al. 2001; Tamekuni et al. 2009). It has also been suggested that MSP1a helps protect the bacterium to evade the host immune system (Garcia-Garcia et al. 2004). In *A. phagocytophilum*, the major surface protein is expressed at different times in its life cycle and is possibly associated with infectivity (Granquist et al. 2008). Studies have also shown that surface proteins play an important role in facilitating the attachment of pathogenic bacteria to their host cells. This has been observed for pathogenic species such as the bacteria *Ehrlichia ruminantium*, *E. chaffeensis* and members of the genus *Rickettsia* (Popov, Yu, and Walker 2000; José de la Fuente et al. 2004; Renesto et al. 2006).

The main surface protein of *Wolbachia* is the WSP (*Wolbachia* Surface Protein). *Wsp*, which was discovered more than twenty years ago, has two transmembrane regions with a strong preference for an outside-inside orientation; suggesting that it may facilitate communication between *Wolbachia* and host cells. In agreement with this prediction, the amino acid sequences of different *Wolbachia* strains cluster in a manner reminiscent of the bacterial distribution of the same *Wolbachia* strains during ovulation of the host species (Veneti et al. 2004), and many of these hypotheses were confirmed by different labs (Melnikow et al. 2013). Furthermore, studies have shown that WSP is a fast evolving component and under strong positive selection while two functional paralogs have also been found in several *Wolbachia* genomes. For all these reasons *wsp* has been used extensively for phylotyping and especially for distinguishing closely related strains (Wangkeeree et al. 2021; Baldo et al. 2006; Baldo and Werren 2007; Kawasaki et al. 2016).

Considering the prominent roles of surface proteins of *Wolbachia* close relatives, I examined:

- a The use of the *wsp* gene as a molecular marker for the identification of *Wolbachia* strains found in the *Anastrepha fraterculus* species complex.
- b Insect host proteins that may interact with the major surface protein of the bacterium *Wolbachia* (*Wsp*) using the yeast-2-hybrid system (Y2H).

C The expression of surface proteins of the bacterium *Wolbachia* (Wsp, WspB, WspC, Sarp, Sa and Sprp) in developmental stages of five different symbiotics *Drosophila-Wolbachia* relations

d The expression and distribution of surface proteins of the bacterium *Wolbachia* (Wsp, WspB and WspC) fused with a GFP (Green Fluorescent Protein) construct in different eukaryotic cell lines.

The first part of this chapter of my thesis focused on *Anastrepha* flies, a group of an insect species complex with a widespread distribution and of great economic importance, making them ideal candidates for potential future biocontrol applications. I contributed to a study of laboratory strains of *Anastrepha fraterculus* (*Tephritidae*) from Peru and Argentina (Cáceres et al. 2009), focusing on the identification of *Wolbachia* strains in this host as *Wolbachia* bacteria had been detected in these flies and were suggested as a cause of reproductive isolation (Selivon, Perondini, and Morgante 1999; SELIVON et al. 1996; 2002). I was responsible for: (a) the identification of *Wolbachia* strains by PCR, RFLP and cloning of the *wsp* gene for Sanger sequencing, (b) the alignment and phylogenetic tree construction, which further contributed to the combined results, which indicated that the two *Wolbachia* two strains belong to different biological entities within the proposed *A. fraterculus* complex.

The remaining parts of this chapter were related to deciphering the molecular functions of *Wolbachia* surface proteins. I initially attempted to identify insect host proteins that may interact directly with the WSP protein. As mentioned earlier, the transmembrane domain of WSP, its fast evolution and the almost perfect prediction of bacterial distribution in host eggs (Veneti et al. 2004) have suggested that it may interact with host proteins. Through cloning of the gene coding the Wsp protein («bait») and a cDNA expression library of *Drosophila melanogaster* (representing the total of insect host expressed proteins) in yeast cells, it was possible to examine approximately 20 million potential interactions. The Y2H system, that had been successfully used to identify proteins of the plant pathogen *Agrobacterium tumefaciens* in the *Arabidopsis thaliana* model, failed to successfully identify any candidate host proteins that interact with the WSP. Some of the potential reasons that the Y2H did not succeed in identifying interactions with Wsp may include: i) a more complex network of interactions than we could not have anticipated; e.g. the possible interaction of Wsp with other bacterial proteins which act as mediators between Wsp and host proteins and ii) limitations of the Y2H technique when it comes to finding specific interactions. For example, publications have reported that the Y2H hybridization method cannot detect interactions when inner membrane proteins or transcription factors are involved (Möckli, Deplazes, and Auerbach 2008).

For the next part of this chapter, with the help of my collaborators I examined the wMel & wRi genomes of *Wolbachia* strains as well as a number of *Anaplasma* and *Ehrlichia* genomes in order to compare and identify genes coding for *Wolbachia* surface antigens. Intracellular bacterial surface proteins play important roles in their biology (Lin, Huang, and Zhang 2002). The *Wolbachia* genome revealed a large number of genes coding for surface proteins (Wu et al. 2004; Foster et al. 2005; Klasson et al. 2008; 2008), that had been very little studied in *Wolbachia* and for this part of the thesis I characterized the six most promising candidates: the *wsp* and two *wsp* paralogs; the *wspB* and *wspC*, and three more predicted surface antigens; the *sarp* (surface antigen related protein), *sa* (surface antigen) and *sprp* (surface protein related protein). I followed two experimental approaches to characterize potential functional roles of these antigens:

i) Using eleven *Drosophila* lab reared lines that were infected with different *Wolbachia* strains, I prepared cDNA libraries from different developmental stages and different insect tissues and examined the expression

of the six genes coding the selected surface antigens. The eleven symbiotic pairs were chosen because the *Wolbachia* bacteria induce different phenotypes ranging from strong CI to no reproductional phenotypes and the bacterial cells have different distributions in the developing eggs and the somatic and reproductional tissues (Veneti et al. 2004).

ii) I cloned the genes coding for the six surface proteins into vectors that allowed the joint expression of the protein of interest fused with a GFP in eukaryotic cell lines. This allowed me to overexpress the proteins of interest in two insect cell lines (S2 and SF9) and two additional mammal cell lines Hela and NIH-3T3. Even though our primary interest has been the heterologous expression of the proteins of interest in insect cell lines, identifying subcellular localization patterns in these cell lines is extremely tedious as their small size and morphology make this task almost impossible. Therefore, the additional mammal cell lines served as controls, especially since many of the eukaryotic proteins of interest (that our focal proteins may interact with; e.g. cytoskeletal proteins such as actin) are highly conserved between insect and mammals.

Selection analyses showed that all six predicted genes appear to be under high positive selection, suggesting prominent roles in *Wolbachia*'s biology. The results of this chapter showed that none of the antigens' expression across different tissues appears to be correlated to the reproductional phenotypes *Wolbachia* causes, but some of them may play important roles in regulating the bacterium's distribution across the host. The genes coding for the Sa and Sprp antigens are not expressed in the wRi strain of subgroup A but are exclusively transcribed and in very high levels in the body tissues of the wMa, wNo and wMau strains, *Wolbachia* strains that have a strong preference for the somatic tissues of the insect host. Furthermore, the heterologous expression of the predicted surface antigens I investigated also showed that: i) the major surface protein Wsp may interact with the microtubules network of the eukaryotic host cell and ii) the *Wolbachia* WspB surface protein may induce programmed cell death to prevent host immune responses.

***Wolbachia* Ankyrins**

Intracellular pathogenic bacteria such as *Chlamydiae*, *Rickettsiae*, *Ehrlichiae* and *Wolbachia* have small genomes. Therefore, they retain only genes that are necessary for their survival and transmission (Palmer 2002). *Wolbachia* genome sequencing and comparative genomics revealed an unusually large number of genes coding proteins containing ankyrin motifs. These patterns are often found in proteins of eukaryotic organisms and viruses (J. Schultz et al. 1998). In fact, more than 3600 different proteins with ankyrin motifs have been identified, but very few have been found in bacteria (Mosavi et al. 2004). The presence of 23 genes coding ankyrins in the wMel strain (Wu et al. 2004), 39 genes coding ankyrins in the wRi strain (Klasson et al. 2009), 12 genes coding ankyrins in the wBm strain and 60 genes coding ankyrins in the wPip strain (2-4 % of total genes) is noteworthy considering that the closely related bacterial species of the *Rickettsia*, *Anaplasma*, and *Ehrlichia* genera have in their genomes very few genes coding ankyrins (Wu et al. 2004; Foster et al. 2005; Siozios et al. 2008; Klasson et al. 2008; 2009). For example, in the pathogenic bacterium *Anaplasma phagocytophilum* HZ there are four genes coding ankyrins, in the bacterium *Ehrlichia canis* Jake there are five while in the bacterium *Rickettsia conorii* Malish there are seven genes (Ogata et al. 2005; Hotopp et al. 2006, 2006; Mavromatis et al. 2006). Thus, the existence of a large number of genes coding ankyrin-like proteins is considered paradoxical given the small genome size of *Wolbachia* (Wu et al.

2004; Foster et al. 2005; Klasson et al. 2008; 2009), and it has been suggested that they may facilitate the *Wolbachia* host interaction.

I initially examined the distribution of 35 predicted genes with ankyrin motifs identified in the *wRi* genome (Klasson et al. 2009) in the *Wolbachia* strains of the same eleven lab reared *Drosophila* lines that were also used for the investigation of surface proteins (see previous chapter). To achieve that I used targeted PCR and southern blot analysis. Subsequently, using a similar approach to the one I used for the study of surface antigens, I investigated the expression profiles of the 35 predicted genes that encode ankyrins (discovered in the *wRi* genome) in the same eleven symbiotic *Drosophila-Wolbachia* relationships. Finally, fused ankyrin GFP constructs were prepared and expressed in insect and mammalian cell lines that allowed me to observe the ankyrins' subcellular localization. Similar to the study of *Wolbachia* surface proteins, I examined distribution patterns and expression profiles of the genes of interest in various *Wolbachia* strains that establish different tissue distribution patterns (e.g. *wMel Wolbachia* cells are solely found in reproductional tissues, *wMa*, *wMau* in somatic tissues while *wRi* cells, which heavily infect their *Drosophila* host, are found throughout the body of their *Drosophila* host) and present a gradient of intensity in the reproductional phenotypes they induce (e.g. *wRi* induces heavily CI cause offspring mortality up to 100% while *wHa* causes almost undetectable CI).

Significant differences were found in the distribution patterns of *wRi* ankyrins in the eleven *Drosophila Wolbachia* symbiotic relationships. Eleven genes were found only in the *wRi* strain and in no other subgroup A strain, while two genes were found in the *wNo*, *wMa* and *wMau* strains but were absent in the *wMel* strain. These patterns correlate perfectly with the different infection and CI phenotypes intensity that *Wolbachia* strains induce in these symbiotic relationships; *wRi* is heavily infected but the rest of strains are not, *wNo*, *wMa* and *wMau* strains are the only ones that are able to infect *Drosophila* somatic cells.

Genes coding ankyrins also presented a variety of expression patterns depending on the life stage of the host; *WRi_006810* was not expressed in 2 hour-old embryos, *WRi_006870* was not expressed in embryos and *WRi_p07650* in 16 hour-old embryos. All three genes belong to the group of "unique" *wRi* ankyrin genes. Sex-specific differences in the expression of the *WRi_003070* gene were detected, as expression was detected in females and ovaries but not in males and testes. In addition, the *WRi_003070* gene has two transmembrane regions (Klasson et al. 2009) suggesting that it may be in contact with host cells. Therefore, we tentatively suggested that the *WRi_003070* may be involved in some bacterial-related mechanism in females, such as the rescue mechanism of CI (the ability of *Wolbachia* to restore synchrony in the mitotic divisions of paternal and maternal chromosomes and thus prevent embryo lethality) and/or the maternal transmission of the bacterium (Klasson et al. 2009).

To examine the potential phenotypes the expression of *Wolbachia* ankyrins may cause to the host, I observed the expression of *wRi* ankyrins in insect and mammalian cell lines. GFP hybrids were constructed and overexpressed in S2 (*D. melanogaster*), SF9 (*Spodoptera frugiperda*), C6/36 (*Aedes albopictus*), Hela (*Homo sapiens*), NIH-3T3 (*Mus musculus*, fibroblasts) and Raw 264.7 (*Homo sapiens*, macrophages) cell lines. A few GFP-fused ankyrins expressed the recombinant proteins primarily in the nucleus and to a lesser extent in the cytoplasm giving a distribution pattern similar to that of the GFP protein (control experiments). These localization patterns suggested that these proteins do not interact with any cell proteins but served as excellent additional negative controls for this study.

Cells transfected with plasmids expressing WRi_003040-GFP WRi_004040-GFP and WRi_006900-GFP as hybrids provided a distribution pattern similar to that of actin. Expression of the WRi_006810-GFP protein was strongly observed around the nucleus and in microtubule-like structures. Ankyrins are a group of proteins that can interact with elements of the cytoskeleton such as actin or microtubules (Bennett & Davis 1981; Sedgwick & Smerdon 1999; Rubtsov & Lopina 2000; Mosavi et al., 2004). At the same time, previous studies have suggested that *Wolbachia* may use the host cytoskeletal components to survive, multiply, and induce reproductive parasitism (Veneti et al., 2004; Ferree et al., 2005; Serbus & Sullivan 2007).

Of interest in the present study, was the morphology of the cell nucleus transfected with plasmid vectors expressing the proteins WRi_001790-GFP, WRi_006740-GFP, WRi_006810-GFP and WRi_006860-GFP. The cells that expressed the above proteins had decondensed nuclei. In addition, when WRi_006740-GFP and WRi_006860-GFP were expressed, the cells had multiple nuclei, suggesting that cytokinesis may have stopped. The above phenotypes indicated that these ankyrins may interact with cytoskeletal proteins and may be able to induce chromatin decondensation. In addition, when I examined the morphology of the cell nucleus of C6/36 cells, infected with *Wolbachia*, nuclei decondensation and interruption of cytokinesis was observed again at a higher rate than normal in infected cells, while these phenotypes were never observed in uninfected cells. It is therefore possible that *Wolbachia*, through the interaction of ankyrins with host proteins, is able to affect the structure of chromatin and cell division of the host cells. Also, as mentioned before, in studies using *Nasonia* and *Drosophila* embryos infected with *Wolbachia*, it was shown that the paternal chromosomes fail to decondense properly and fuse with the parent chromosomes before entering the first mitotic division, which leads to embryonic mortality (Lassy and Karr 1996; Callaini, Dallai, and Riparbelli 1997; Tram and Sullivan 2002). This led us to believe that *Wolbachia* ankyrins may be involved in CI through interaction with chromatin or other cell protein(s) that control its decondensation. In addition, it is worth mentioning that in the pathogenic bacterium *Ehrlichia phagocytophilum*, a phylogenetically close relative of *Wolbachia*, is able to secrete the AnkA ankyrin, which interacts with the chromatin of the host cells (Caturegli et al. 2000).

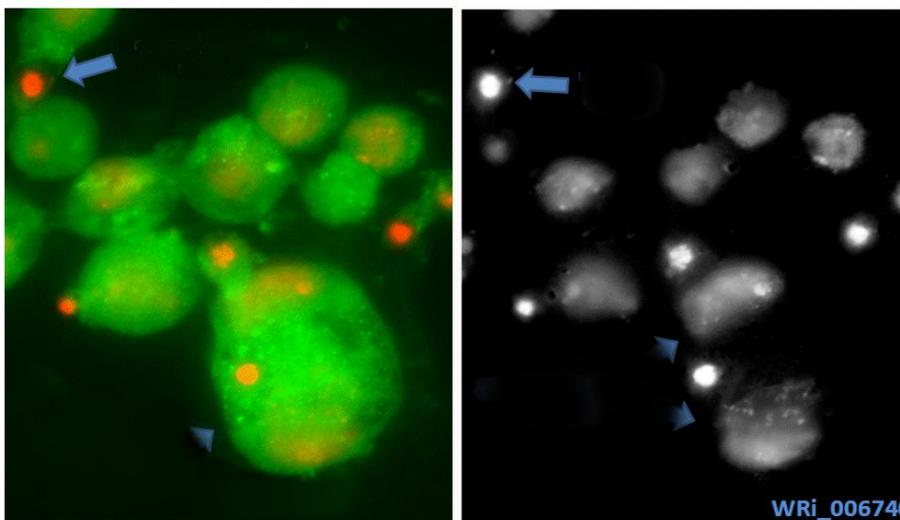


Figure 1: Ank-GFP overexpression in S2 cells.

To the left pseudocolors have been assigned to differentiate staining of the ank-GFP (green) and DNA staining (red; propidium iodide). The example presented here shows the chromatin decondensation that was observed when the fused «006740» ankyrin was expressed in S2 cells. Arrows on top show a normal cell,

arrowheads on the bottom show the decondensed nuclei of a cell expressing the WRi_006740 protein.

Investigation of the role of WO-B bacteriophage.

A bacteriophage is a virus that infects bacteria. The first evidence of a bacteriophage infecting *Wolbachia* was presented in 1978, when Wright and his colleagues observed VLPs (Virus Like Particles; (Wright et al. 1978). These particles were similar in size and shape to the hitherto known phage capsules and were detected only in *Wolbachia* cells. Sequencing of *Wolbachia* genomes revealed multiple prophage regions in various *Wolbachia* strains; wMel has three prophage regions ; WO-A, WO-B and a small pyocin-like element (Wu et al. 2004), five such regions (closely related to the bacteriophage WO-B) were found in the wPip genome (Klasson et al. 2008) and four prophage regions in the wRi genome (Klasson et al. 2009). Interestingly, bacteriophage regions were not found in the wBm genome or in other *Wolbachia* strains infecting filarial nematodes, which have established mutualistic relationships with the host (Foster et al. 2005). Therefore, since bacteriophages have only been found in *Wolbachia* strains that have developed a parasitic relationship with their arthropod hosts, it has been suggested that WO bacteriophages may be involved in the induction of reproductive abnormalities (Wu et al. 2004; Klasson et al. 2009).

Among the various bacteriophage genes that have been suggested to play a role in inducing *Wolbachia* reproductive abnormalities are the methylase genes. The first report of the presence of a methylase gene in *Wolbachia* was in 2000. The gene was found in the bacteriophage WO genome region of the wTai strain (Masui et al. 2000). Genome sequencing of wMel confirmed the presence of a methylase gene in the WO genomic region (Wu et al. 2004).

On the other hand, a study on *N. vitripennis* infected with *Wolbachia* suggested a very different relationship between *Wolbachia* and the bacteriophage WO-B. The bacteriophage WO-B appeared to be active, to form VLPs, and to lyse *Wolbachia* cells (Bordenstein et al. 2006). More specifically, 11.8% of *Wolbachia* bacteria cells appeared to be in the lytic phase (Bordenstein et al. 2006). The results of this study led to the theory of the inverse relationship between density of the bacteriophage WO-B and *Wolbachia* density. Therefore, the authors suggested that the bacteriophage prevents *Wolbachia* from causing CI because the CI levels are proportional to the number of *Wolbachia* cells in the testes (D. Poinot et al. 1998; Clark et al. 2002; Denis Poinot, Charlat, and Merçot 2003; Clark et al. 2003; Veneti et al. 2003). In the same study, the authors implied that the bacteriophage WO-B was inversely associated with the induction of reproductive parasitism by *Wolbachia* as only bacterial cells that did not contain VLPs were in contact with spermatids (Bordenstein et al. 2006).

This chapter of my thesis investigated the WO-bacteriophages in *Wolbachia*, both assuming that they may promote reproduction phenotypes and thus aid *Wolbachia* to its parasitic lifestyle, but also entertaining the alternative hypothesis that they may have a detrimental effect on *Wolbachia* cells. To achieve that I examined: (a) the expression of two DNA-methylase genes that occur in the WO-A and WO-B bacteriophage regions in different tissues and developmental stages in 11 *Drosophila-Wolbachia* symbiotic relationships, and (b) the WO-B Orf7 capsid protein distribution in different *Wolbachia*-arthropod symbiotic relationships, using a polyclonal antibody constructed for this study. For the latter, I examined the distribution of the minor capsid protein of the WO-B bacteriophage and the distribution of *Wolbachia* major surface protein (Wsp) by Western Blot analysis and performed immunoprecipitation. In addition, I also examined their tissue localization using confocal fluorescence microscopy in different *Drosophila* tissues.

For the DNA methylase genes, the molecular and phylogenetic analysis identified two slightly different copies of the gene, *met1* and *met2*, that exhibit a different distribution over various *Wolbachia* strains, but no correlations between CI phenotypes and the distribution or the expression of these genes were found (Saridaki et al. 2011).

Regarding the second part, the anti-Orf7 antibody was used to analyze the distribution of the WO-B capsid protein in different insect-*Wolbachia* symbiotic associations by Western blot and immunofluorescence confocal microscopy. The initial checks on the purified antibody showed that the anti-ORF7 antibody detected extracellular phage particles enriched from insect homogenates of *Wolbachia*-infected hosts. I then used the Ab to examine the bacteriophage distribution in *Drosophila* tissues, which showed that there were obvious differences between *Wolbachia* cells and the bacteriophage distribution in *Drosophila* testes and ovaries, including a notable differential localization pattern between phage and *Wolbachia* cells during mid-oogenesis and the potential presence of bacteriophage VLPs in the cytoplasm of spermatocytes. The overall results of this chapter suggested that the WO-B bacteriophage lyses *Wolbachia* cells infecting *D. melanogaster* and thus the phage may have a functional role in the *Drosophila Wolbachia* symbiosis during early sperm and egg development. An anti-ORF7 antibody could have been a valuable tool for characterizing the life cycle, distribution, and function of *Wolbachia* bacteriophage. However, this study was never followed up because the size variance of the WO-B antigen (western blot gels) and the inability to sequence the identified peptides, made it impossible to verify the above findings and the specificity of the antibody.

More general implications of my PhD work for other research subjects

My *Wolbachia* work has additionally made important contributions to a number of other studies as I used my skills and expertise to contribute to a number of other projects that involved interdisciplinary collaboration with other labs. The bullet points below summarize them:

I contributed to a study investigating the origin of replication in the *Wolbachia* genome and its close *Rickettsia* relatives. In this study, we showed that *Wolbachia* and its close relatives may have altered replication mechanisms, but the implications of this finding in the biology of *Wolbachia* remain unclear. I was responsible for the PCRs, nested PCRs and cloning for sequencing of the ori gene fragments that we analyzed in this study (Ioannidis et al. 2007).

I contributed to an MLST study of *Wolbachia* strains with the objective of determining the root of the *Wolbachia* phylogenetic tree. In this project I was responsible for: (a) the PCR, nested PCR and cloning for sequencing analysis, (b) the nucleotide analysis, including alignments and the concatenated alignment of the sequences. This collaborative project was pioneering in highlighting the potential of phylogenomics to resolve complicated phylogenies (Bordenstein et al. 2009), which was confirmed a few years later (Gerth et al. 2014).

I contributed to the investigation of the gut microbial community of *Bactrocera oleae* (*Tephritidae*), a major insect pest (Kounatidis et al. 2009). Using 16S generic PCRs and cloning of the amplified 16S fragments I contributed to the Sanger sequencing and characterization of the *Bactrocera oleae* gut microbiota.

Post-doctoral position in the BF2I laboratory, Lyon, France (2009 -2011)

After finishing my PhD, I moved to France to work on the model trophic symbiosis between *Buchnera* and the pea aphid (*Acyrtosiphon pisum*). This was my first postdoctoral experience and took place in the Laboratory "Biologie Fonctionnelle, Insectes et Interactions" (BF2I) from March 2009 till April 2011.

Buchnera aphidicola bacteria are close relatives of the free-living *Escherichia coli*, but approximately 150-200 MYA (Moran et al. 1993), *Buchnera* formed an association with the aphids and natural selection reshaped the genomes of these bacteria, resulting in the loss of all genes necessary to sustain themselves, and making them entirely dependent on their host for nutrients and protection. The dietary preference of the aphids cemented this relationship, as their symbiotic association is based on the exchange of essential and non-essential amino acids (Brinza et al. 2009; Douglas 1996).

The project I was involved focused on the identification of host genes regulating the symbiotic response to nutritional stress. My part in this project was the development of an RNAi technique to inactivate aphid gene targets. More specifically, I performed: i) a systematic comparison of two different dsRNAi administration methods: microinjection and feeding, and ii) the analysis of the knockdown and phenotypic effects of dsRNAi in each separate body compartment (bacteriocytes, gut, embryos, head and the remaining carcass) of single individual aphids. The most important findings of this project were: i) the identification of the RNAi knockdown distribution in the pea aphid body and ii) the morphological phenotypes in the pea aphid as a result of the cathepsin-L inhibition. During this period, I was responsible for establishing the optimal RNAi delivery method to maximize efficiency of any given gene expression knockdown and turn the methods I used into an RNAi platform that the technical personnel of the Bf2i lab (TAPs Gabrielle duport et Karen Gaget) could easily apply to the study of any aphid genes. With that goal I trained the two technicians in the use of the technique and the Bf2i lab has successfully used the RNAi inactivation pipeline for several projects (Simonet et al. 2018; Simonet, Duport, et al. 2016). As part of this project I also supervised François Cochet, an M1 intern (« stagiaire »). During his internship, I was responsible for supervising François in the molecular biology lab where he performed RNA extractions from aphid tissues and performed quantitative PCR on a range of aphid genes to quantify and compare their expression. My supervision included the planning of his work and the day to day experiments, the tutoring in molecular biology techniques and the discussion and interpretation of results.

I was also involved in a transcriptomic study that examined the potential regulation of amino acid related genes during the aphid development (Rabatel et al. 2013). My involvement was both in the experimental work and the analysis and interpretation of the results.

Post-doctoral researcher at the Centre for Social Evolution (CSE), Copenhagen, Denmark (2011-2019)

Following my brief postdoctoral period in France, I moved to Copenhagen (Denmark) to join a highly interdisciplinary team composed of evolutionary and molecular biologists, ecologists, biochemists, statisticians and other researchers from various backgrounds. There, I was at the center of the microbiota research accomplished by CSE (a former Center of Excellence supported by Danmarks Grundforskningsfond for 10 years) and stayed for almost eight years. When I was hired, initially supported by an individual EU Marie Curie Fellowship, CSE's microbiology programs (Professors Boomsma and Poulsen) had just started, and I had a major role in their successful progression, both as lead-scientist with independent expertise from my training in Greece and France, and as mentor and co-supervisor. I established the platform for the 16S-Miseq that was being used at the Centre until recently and trained undergraduate, MSc and PhD students as well as postdoctoral researchers to perform wet lab experiments and sequencing for the characterization of the gut microbiota of various model systems. My primary research task was the identification and functional characterization of bacterial gut symbionts of fungus-growing ants and fungus-growing termites.

Fungus-growing ants

The main model system that I built my research during my period at CSE was the fungus-growing ants. Fungus-growing ants are a model system of a complex symbiosis because these ants were the first agriculturists on our planet as they switched from a hunter gatherer lifestyle to an agricultural one approximately 50 million years ago (T. R. Schultz and Brady 2008). These ants cultivate in monocultures a domesticated strain of fungus and they protect it from external pathogens and diseases with the use of elaborate social behaviors and antibiotic producing *Actinomyces* bacteria that they cultivate on their cuticle. The FG ants tribe (attines) is composed of seventeen currently recognized genera (Sosa-Calvo et al. 2013; 2018) and based on the form of agriculture they practice, it is believed that FG ants underwent two major transitions during their evolutionary history: one that took place ca. 25–30 MYA, starting with the permanent domestication of a single fungal lineage that produced specialized hyphal tips (gongylidia) to feed the ants (evolution into monocultures), and another ca. 15–20 MYA when the leaf-cutting ants arose as active herbivores and evolved industrial-scale farming (Currie et al. 2006; Kooij et al. 2015; Ulrich G. Mueller et al. 2005; U. G. Mueller, Rehner, and Schultz 1998; Nygaard et al. 2016; T. R. Schultz and Brady 2008; Shik et al. 2014; Villesen et al. 2002). As a result of that, FG ant species have very different lifestyles and diets: the least evolved species at the bottom of the phylogeny (lower attines) have small sized colonies (often <50 workers), forage dead material (e.g. insect carcasses) and rotten wood and have no specialized castes, while the most evolved species (a.k.a. leafcutters that transitioned to industrial farming) forage fresh plant material (e.g. leaves, flowers), have often specialized castes (i.e. workers and soldiers of different sizes) and can have colonies of many millions (Currie et al. 2006; Kooij et al. 2015; Ulrich G. Mueller et al. 2005; U. G. Mueller, Rehner, and Schultz 1998; Nygaard et al. 2016; T. R. Schultz and Brady 2008; Shik et al. 2014; Villesen et al. 2002). Therefore, the various transitions from lower to higher attines and leafcutters over the course of evolutionary time have resulted in many adaptations to the ant host, the fungal cultivar and the bacterial communities associated with the ants or the fungus. The complexity of the fungus growing ant

system is therefore the reason that it is an excellent model to study as their societies can be used as an analogy to different stages of human agricultural societies.

When I arrived at CSE in 2011, the gut bacterial communities of fungus growing ants had been very little investigated (Van Borm, Billen, and Boomsma 2002) and therefore my main project at CSE was their identification and characterization. I took an integrated approach to identify and characterize them taxonomically, place them phylogenetically, use ecological approaches to correlate factors that affect their abundance and prevalence, unravel their tissue localization but also understand their function in the fungus growing ant symbiosis.

Identification of gut bacteria in leafcutter ants and factors affecting their prevalence and abundance

I initially used targeted 16S metagenomic sequencing and FISH microscopy to examine the gut bacteria of *Acromyrmex* fungus growing ants, an attine species that is considered leafcutter and even though it does not have specialized castes it can form colonies of millions of workers. My study (AEM) showed a consistently across colonies low diversity community of Mollicutes, Rhizobiales and *Wolbachia*. Considering the meticulous strictly liquid diets of *Acromyrmex* ants (leafcutters), a low diversity gut bacterial community was not a surprise because typically the more specialized diets tend to reduce diversity in gut microbial communities; e.g. honeybees and bumblebees have microbiota dominated by rather few bacterial species, consistent with bees having more predictable pollen and nectar diets than ants and termites, which have generalist feeding ecologies (Koch and Schmid-Hempel 2011a; Engel, Martinson, and Moran 2012; Koch and Schmid-Hempel 2011b; Kwong et al. 2014; Martinson et al. 2011). Furthermore, considering that pollen is rather protein rich (Campos et al. 2008) relative to leaves (Quinlan and Cherrett 1979) which *Acromyrmex* ants chew but only swallow the liquid part, we would expect the leafcutter ant microbiota to have a higher likelihood of providing nutritional supplementation. Taken those facts into account I examined whether some of these symbionts may aid to nitrogen supplementation as a result of these protein poor diets. Using *nifH* specific PCRs, NifH antibody immunohistochemistry and electron microscopy my colleagues and I showed that Rhizobiales symbionts inhabit the *Acromyrmex* hindgut as a biofilm, and fix (probably atmospheric) nitrogen-just like congeners that abide in root nodules of legumes on human farms. The biofilm structure makes these symbionts especially resilient as when the entire bacterial community was challenged with a range of antibiotics, the biofilm forming Rhizobiales were less vulnerable than some of the other taxa (Sapountzis, Zhukova, et al. 2015).

Following up the insights in the *Acromyrmex* fungus-growing (FG) ant (which is one of the most evolved species in their phylogeny; leafcutters), I expanded the comparisons across gut microbiomes in the entire phylogeny of the FG ants. In order to understand how gut bacterial communities of FG ants were shaped and which factors affect them, I examined gut bacterial communities change across branches of the host phylogeny and shifts in host diets a comparative microbiome analysis of endosymbiotic bacteria spanning the entire phylogenetic tree. I showed that, across 17 representative sympatric species from eight genera sampled in Panama, abdominal microbiomes are dominated by Mollicutes, α - and γ -Proteobacteria, and Actinobacteria. Bacterial abundances increase from basal to crown branches in the phylogeny reflecting a shift towards putative specialized and abundant abdominal microbiota after the ants domesticated gongyldia-bearing cultivars (higher attines), but before the origin of industrial-scale farming based on

leaf-cutting herbivory (leafcutters). This transition that our study identified coincided with the ancestral single colonization event of Central/North America ca. 20 MYA, documented in a recent phylogenomic study showing that almost the entire crown group of the higher attine ants, including the leafcutter ants, evolved after moving to this new area (Branstetter et al. 2017). Several bacterial species are located in gut tissues or abdominal organs of the evolutionarily derived, but not the basal attine ants.

Finally, the very same study showed that the composition of the ant gut microbiomes appears to be affected by the presence/absence of defensive antibiotic-producing actinobacterial biofilms on the worker ants' cuticle (Sapountzis et al. 2019), but the significance of this association remains unclear.

Rhizobiales bacteria

Following an early pilot experiment during which it was shown that several gut bacterial taxa appear more resilient than others in the presence of antibiotics (Sapountzis, Zhukova, et al. 2015), I set out to investigate potential phenotypic effects in the ants when their gut symbionts were removed. This experiment was part of an undergraduate's student short project («fagprojekt»), Anna Fomsgaard who stayed for an internship at the centre for 3 months. During that project Anna, under my direct supervision, isolated *Acromyrmex* ant workers in sterile petri dishes and fed them for a period of ten days either a mixture of sucrose water with tetracycline and rifampicin or sucrose water without antibiotics (control). The isolated ants were monitored daily for phenotypic changes and mortality counts and at the end of the 10 day period, the ant guts were dissected and DNA was extracted. Bacterial counts were quantified using taxa specific primers and qPCR and community changes were evaluated using 16S-Miseq sequencing.

This work confirmed previous findings that had suggested a slightly higher mortality when Rhizobiales bacteria are removed from the gut, suggesting that this taxon may be essential for the ant survival and it also served multiple other functions: i) the protocol that we used to isolate and feed ants artificial diets served as a pipeline that was used for several subsequent similar experiments, ii) some of the results of this experiment were a stepping stone for Anna's BSc thesis.

Following up on the Rhizobiales hypotheses regarding their importance in the fungus-growing ant symbiosis, postdoctoral researcher Mariya Zhukova and I isolated Rhizobiales bacterial cells using two different methods: i) using direct microdissections to excise the part of the gut that Rhizobiales are located, and ii) using anaerobic *in vitro* cultures that allowed them to grow (albeit in a very slow rate). Material from both methods was sent for genome sequencing and the assembled genomes as well as several closely related Rhizobiales genomes were used for comparative genomics. The results of this study suggested that Rhizobiales gut symbionts may play a role in nitrogen recycling in the ants gut by catabolizing excess of fungus-garden-derived arginine to urea, and thus supplementing complementary Mollicutes symbionts (see next page). Since a similar function has been suggested for Mollicutes this would imply that this functional redundancy may be a part of a larger failsafe system that ensures the stability of the fungus-farming mutualism (Zhukova et al. 2022).

Mollicutes endosymbionts

Having discovered that Mollicutes (order: Entomoplasmatales) bacteria are a crucial component of the most evolved FG ant lineages (Sapountzis, Zhukova, et al. 2015; Sapountzis et al. 2019), I set out to investigate their functional roles using *in silico* and wet lab approaches. I isolated Mollicutes bacterial cells (single strains verified by PCR) from the fecal droplets of *Acromyrmex* FG ants (leafcutters) and fat body cells of *Apterostigma* FG ants (lower attines) by adapting a pipeline that had been used for isolating *Wolbachia* and other insect endosymbionts. Then, using Multilocus Displacement Amplification and genome sequencing I produced the genomes from the two most common Mollicutes endosymbionts associated with FG ants: the highly specialized and abundant in leafcutter (most evolved) lineages *EntAcro1* (isolated from *Acromyrmex*) and the non specialized and sparse *EntAcro10* (isolated from *Apterostigma*). Using the sequences of the identified strains, I initially performed phylogenomics using all available Mollicutes genomes at the time, functional annotation and comparative genomics to identify metabolic or other pathways that were unique in the two strains. Then I performed correlations between the functional annotations, the abundances of the bacterial cells in ant tissues and the components of the ant diets. Finally, I confirmed a few of the hypotheses the genomic comparisons revealed using molecular approaches. Our study showed that both Mollicutes strains have the potential to turn excess quantities of the amino acid arginine into a nitrogen-rich fertilizer the ants deposit on their fungal gardens as feces. However, only the specialized *EntAcro1* strain (associated with the leafcutters) can decompose citrate from plant sap and fruit juice (consumed by the leafcutter ants) and produce acetate, a common energy source that often gut bacteria produce for their hosts, which supplements the ants' fungal diets and provides extra energy (Sapountzis et al. 2018). The findings of this study added an extra layer to the already complex FG ant symbiosis by revealing how the gut bacterial symbionts of these ants contribute too towards the stability of the farming lifestyle of their ant hosts.

In parallel with the above project, Anna Fomsgaard who had done a short internship in 2015 came back to the centre to continue on the work that she had started before. Under my supervision, Anna examined three different hypotheses related to the functional roles of *EntAcro1* bacteria in *Acromyrmex* FG ants:

- i) the metabolic role of *EntAcro1* across different colonies, by examining the transcription levels of 15 *EntAcro1* genes in various ant tissues: four chitin-related genes, four genes used in arginine metabolism and seven genes used for citrate utilization,
- ii) the potential defensive functional role of *EntAcro1* by monitoring changes in the ants' phenotype (including mortality) when removing the Mollicutes with antibiotics and exposing them to different pathogens, and
- iii) test if the growth rate of the ants' fungus is influenced by the Mollicutes presence in the ants' fecal droplets.

Anna's thesis took an integrative approach to examine all potential hypotheses that we had formed up to that moment regarding the role of *EntAcro1* in the FG ant symbiosis. By examining the transcription levels of genes related to the metabolic potential Anna showed strong expression of these genes in the guts and fecal droplets of the ants suggesting that the Mollicutes are mainly in the hindguts and are excreted with the fecal droplets. Small differences in the expression of the genes were identified (i.e. chitin and arginine related gene expression was slightly higher in fecal droplets while citrate utilization in the guts) but none of these contrasts were significant when formally tested, even though the numerical contrasts were consistent with

our hypotheses about citrate utilization and arginine recycling. Following up on the results of the transcription, we then examined whether fecal droplets from ants would enhance the growth of fungus monocultures however, there were significant difficulties into isolating the correct strain of fungus cultivar and keeping it clean from infections as one of the most important reasons that the fungus stay clean is the constant meticulous cleaning by the workers.

Anna's thesis also examined the potential of Mollicutes bacteria to affect the mortality of ants when infected with selected pathogens. For that purpose two pathogens that FG ants can encounter were selected: the fungal pathogen *Metarhizium* and the opportunistic bacterial pathogen *S. marcescens*. There were no significant differences in the mortality of ants infected with *Metarhizium* when the ants had their Mollicutes symbionts intact or eliminated with antibiotics, which inclines that the Mollicutes are not essential for parasitic fungal removal. However, there was a difference in the mortality of ants infected with *S. marcescens*; The experiment showed that when the ants did not have Mollicutes (because they had previously received antibiotics) the ants had a higher mortality rate. The precise investigation of why Mollicutes had such an effect was not possible within the framework of Anna's thesis, however considering that a prerequisite for *S. marcescens* virulence is to eliminate the native microbiota of the host it infects -which is achieved by using a T6SS that attacks the cell wall of its competitors (Diniz and Coulthurst 2015)-, our working hypothesis was that since *EntAcro1* has no cell wall, it is immune to *S. marcescens* virulence and quickly outcompetes the pathogen.

The intriguing interaction between Mollicutes symbionts and the opportunistic pathogen *S. marcescens* was unfortunately not immediately followed up as Anna left the lab to continue her studies (she left to follow a MSc course in Evolutionary Biology at the UCPH) but also because these experiments required field work, which could only be done in south and central America, where the ants are indigenous. During that time (2017-2018), fulbright MSc student Veronica Sinotte was visiting the lab and intrigued by the project she asked to continue the project. Under Koos Boomsma and my supervision, Veronica made a successful application for a Smithsonian Tropical Research Institute grant in Panama in order to visit the rainforest and collect samples during May–August 2018. However, even though she successfully found *Acromyrmex* ant colonies and isolated *S. marcescens* strains, the colony and experimental set up without the proper lab facilities and equipment (Veronica was located in Gamboa, outside of Panama city) took a very long time and she was not able to perform the planned experiment within the three month period.

***Wolbachia* in FG ants**

As mentioned above, *Wolbachia* is an intracellular maternally inherited bacterium that is able to manipulate its insect host reproduction in order to spread efficiently in insect host populations through females but not males. Inducing reproductive phenotypes with a preponderance of female function aids *Wolbachia* to spread efficiently because it is transmitted maternally (Werren 1997). However, in social insects like ants, only the ant queen can produce offsprings, offsprings that will either become sterile workers that tend the colony or functional reproductive capable queens and males that will fly away to establish new colonies once they reach maturity. Therefore, the presence of *Wolbachia* in sterile ant workers poses an evolutionary paradox as it is a dead end for horizontal transmission. In FG ants, previous work spearheaded by CSE (Andersen et

al. 2012; Sapountzis, Zhukova, et al. 2015; Zhukova et al. 2017) showed that sterile workers have relatively high *Wolbachia* titers in their guts suggesting a trophic role.

We set out to test the hypothesis of a potential trophic mutualism by looking for *Wolbachia* genes known to aid to the nutritional supplementation of their hosts and assuming that they may be functional in *Wolbachia* colonizing ants too. At the time there were already several complete genomes available from other insect hosts where *Wolbachia* complements the host nutrition (e.g. the bedbug *Cimex lectularius*). Vitamin or amino acid supplementation are all credible hypotheses that we aimed to investigate. Comparisons also included the known CI inducers *w*Ri, *w*Mel, and *w*Pip strains as genes causing CI in solitary insects may be relevant to the unique eusocial lifestyle of ants (i.e. genes possibly related or involved in caste differentiation). We isolated and sequenced two novel *Wolbachia* strains found in FG ants. In order to isolate single *Wolbachia* strains and eliminate any contamination by other bacteria present, we collected ant eggs and surface sterilized them as it had already been demonstrated that eggs carry only a single *Wolbachia* strain and no other bacteria (Andersen et al. 2012; Zhukova et al. 2017). This project was spearheaded by me and was carried out with postdoctoral researcher Mariya Zhukova and visiting PhD student Sarah Jo Al Tolley. Sarah visited the centre (2015-2016) to work on this project as part of her PhD. I was her direct supervisor during her visit and I continued to act as an advisor for the duration of her PhD.

Besides obtaining *Wolbachia* genomes for the purpose of performing comparative genomics, we also aimed to obtain high quality transcriptomes to test some of the hypotheses that would derive from the metagenomics analysis. For this part we used two approaches:

i) Analysis of gene expression of genes that are present in *Wolbachia* isolates from ants and absent in *Wolbachia* strains from other insects, which entailed targeted PCR primers and probes and the use of quantitative RT-PCR and microscopy to examine the location of these genes and the number of copy variants.

ii) Comparative metatranscriptomics to identify differentially expressed *Wolbachia* and host genes at each ant developmental stage.

This study provided not only the first ant-derived *Wolbachia* genomes, but also the first insight into the complex relationship between *Wolbachia* and the leafcutter ant, *A. echinator*, a social insect symbiosis that is unexplored yet frequently occurring in nature (Tolley et al.; in prep - to be submitted in 2023). Phylogenomics identified the two ant derived strains as most closely related compared to any other *Wolbachia* genomes and positive selection analyses suggested that the *ribE* (riboflavin synthase) gene may present signatures of positive selection in both the leafcutter ant-associated *Wolbachia* and the mosquito-associated *Wolbachia* strains. *Wolbachia* provides riboflavin to hosts with specialized diets (e.g. the *w*Cle strain associated with the bedbug, *Cimex lectularius*; (Moriyama et al. 2015). Differential expression analyses across ant developmental stages suggested that *Wolbachia* may also be involved in Heme supplementation during development. Our study highlighted that rather than rely on a single process or pathway, this symbiosis may be a symphony of complex interactions that fluctuate over the development of the host and will require extensive future studies to understand the depth of the intertwined interactions between *Wolbachia* and ant hosts (Tolley et al.; in prep - to be submitted in 2023).

In parallel with the above main project Sarah and I worked in two additional smaller projects:

i) We performed a quick analysis to look for *Wolbachia* genes in the ant genome. The genomes of obligately mutual *Wolbachia* strains infecting nematodes are significantly smaller than parasitic strains harbored in other insects. Given *Wolbachia*'s propensity for horizontal gene transfer, we assumed that we may find sections of the *Wolbachia* genome incorporated in the ant genome, which further may be pseudogenized in the *Wolbachia* genomes. Loss of these genomic regions to the host genome would align host and endosymbiont interests thereby ameliorating intergenomic conflict. However, no evidence of *Wolbachia* gene transfer to the *Acromyrmex* genome was found.

ii) The social lifestyle of ants can often attract social parasites. These parasites are found throughout social Hymenoptera (i.e., bees, wasps and ants), and have mainly been studied in ants. In FG ants (similar to other ants) social parasitism forms mixed species nests, which depend on labor provided by the host species workers. The close interactions between the different species within the same nest offer great opportunities for symbiont transfer and considering how *Wolbachia* has been implicated in horizontal transfer throughout many systems, we decided to revisit an old study (Van Borm et al. 2003) that suggested *Wolbachia* horizontal transfer between *Acromyrmex echinator* and its social parasite *Acromyrmex insinator*. During a short trip in Gamboa, Panama, Sarah identified a unique *A. echinator* colony parasitized by three *A. insinator* queens. She characterized the infection status of each queen and the transmission potential of their *Wolbachia* strains by separating them, collecting eggs and Sanger sequencing the fast evolving *wsp* gene of the *Wolbachia* strains. We used the results she obtained to compare them to a study published more than 15 years ago by Van Borm et al. (2003). The results of this pilot study prompt us to reevaluate previous notions that obligate social parasitism reliably facilitates horizontal transfer and suggest that not all *Wolbachia* strains associated with ants have the same genetic potential for horizontal transmission (Tolley, Nonacs, and Sapountzis 2019).

RNAi approaches in ants to study their gut bacterial symbionts

On account of my experience with the RNAi technique (that I had acquired during my short postdoctoral period in France), I was assigned to establish a working pipeline for knockdown of ant genes. At the time, with the exception of one study (Lu, Vinson, and Pietrantonio 2009) there had been no RNAi studies on ants and therefore setting up the technique had a promising pioneering character. This project was also part of a short internship of Oluf Sonne Georg, an undergraduate student in the Department of Biology of UCPH who I supervised in the lab and the writing of his internship report. With Oluf, we administered to *Acromyrmex* ants dsRNA and siRNA constructs targeting the uniformly distributed (throughout the ant body) VacATPase and examined the effects the artificial feeding had in isolated ant subcolonies morphologically and by checking the vacATPase expression. The knockdown efficiency was analyzed by measuring the expression levels in dissected guts of ant workers and the remaining tissues. Two administration experiments were performed: one using vacATPase dsRNA and one using vacATPase siRNA and surprisingly the former seemed to be more efficient than the latter. Unfortunately, the short duration of Oluf's internship and my engagement in numerous other projects at that time did not allow the further investigation and establishment of the RNAi pipeline in FG ants.

The link between gut microbes and ant behavior

A big part of my work at CSE involved the removal of gut microbes, typically by administering a single or a mix of antibiotics. Such experiments took place often and were either part of a student's thesis (e.g. Anna Fomsgaard) or a bigger project (Sapountzis, Zhukova, et al. 2015). In order to administer artificial diets and antibiotics to the ants, I typically separated them in smaller subcolonies where their natural diet regime was replaced by a single liquid diet (sucrose +/- antibiotics). Since the first antibiotic administration experiments I performed, I observed a peculiar phenotype: ants that had ingested antibiotics for several days would become aggressive and fight their own nestmates (i.e. ants coming from the same colony). This was particularly remarkable because ants, as a result of their social lifestyle, communicate with each other using pheromones, sounds, and touch. The use of pheromones as chemical signals is more developed in ants than other insect groups. Ants perceive smells with their antennae and thus possess a fascinating and admirable ability to smell that allows them to tell friends (nestmates) from foes (non-nestmates). The fact that this behavior would only take place in experimental subcolonies that had been fed antibiotics for several days suggested that the removal of gut microbes may have triggered it.

In collaboration with postdoctoral researchers Jelle van Zweden (at the time in the university of Leuven, Belgium), Luigi Pontieri (CSE, UCPH) and Serafino Teseo (NTU, Singapore), we took an integrative approach that combined metagenomic, chemical and behavioral approaches to test whether gut microbes affect nestmate recognition in *Acromyrmex echinator* leafcutter ants.

Using 1500 individual workers from four *Acromyrmex* colonies and setting them up in individual arenas to monitor their behavior and quantify their aggression, we examined how they responded when: i) they came directly from their own colony, ii) had been isolated for two weeks and fed a sterile sucrose diet and iii) had been isolated and fed antibiotics. After the first round of experiments and monitoring of their behavior in the aggression trials, the same ant workers were fed either fecal droplets from their old colonies or from a different colony and the trials were repeated. Each ant worker, whose behavior was evaluated in aggression trials, was dissected and their gut microbiota was examined using 16S-Miseq sequencing and qPCR while the remaining tissues were used to analyze their chemical profile using GC-MS. As expected, treating workers with a sterile diet or with antibiotics resulted in a substantial alteration of their gut microbial communities. In pairwise social interactions, untreated vs antibiotic-treated nestmates behaved more aggressively than other nestmate and non-nestmate pairs, suggesting that the suppression of microbes may alter chemical social cues and trigger aggressive behavior. Chemical analyses on treated individuals revealed a decrease in the abundance of two metapleural gland antifungal compounds, and the dissimilarity between aggression levels and chemical profile covaried significantly. Feeding antibiotic treated ants with fecal droplets partially restored the original bacterial communities. Non-nestmates fed with fecal droplets from different colonies were unusually aggressive compared to pairs fed with fecal droplets from the same colony, suggesting that chemicals derived from or influenced by gut microbial strains may shape nestmate recognition, opening novel questions about the role of microorganisms in the evolution of social behavior (Teseo et al. 2019).

Fungus growing termites

Fungus-growing termites (*Macrotermes* and *Odontotermes* genera) of the worker caste harbor hundreds of bacterial species in their guts. This complexity allows them to degrade foraged substrates and nutrients provided by their fungus symbiont (through gut passage), since the complementary contributions of all symbionts identified in the symbiosis represent 86.7% of all known glycoside hydrolases in the world (Poulsen et al. 2014). This has potentially contributed to the fact that these termites have no known diseases and zero waste (Poulsen 2015).

I was involved in a number of gut microbiota projects in fungus-growing termites, where I participated/spearheaded projects, co-supervised one BSc, two MSc and a PhD student.

As part of Antoine's Frainout MSc thesis, we investigated the antibiotic producing potential of *Bacillus* sp., which was isolated from the fungus-growing termite fungal cultivar. Antoine Frainout, under my supervision, examined the genomes of Bacilli isolated from the fungal cultivar and close bacterial relatives, which showed that they are closely related to *Bacillus subtilis*, the multiple isolates have almost identical genomes and carry genes related to antibiotic producing compounds. The overall results of this work suggested that these antibiotic-producing bacteria may be defensive symbionts of the termites that could protect against fungal pathogens (Um et al. 2013).

As aforementioned, the FG termites harbor a highly complex gut microbiota that allows them to perfectly degrade forage substrates and acquire nutrients. However, this complexity also creates a highly competitive gut environment, which would require bacteria to develop adaptations to withstand it. As a result of the community complexity and because these typically anaerobic bacteria can rarely be cultured, not much is known about their physiological capabilities and their interactions with the termite host. The bacterium *Trabulsiella odontotermis* is known to be associated with fungus-growing termites (Chou et al. 2007), but there had been no studies investigating its function. This project was part of the Thijs Gruntjes BSc thesis (ERASMUS project). Thijs, under my supervision, performed comparative genomics to identify *T. odontotermis* genes that were exclusively (or more frequently) present in strains associated with the FG termite lifestyle & gene expression experiments to quantify their expression. In addition, we also performed FISH microscopy to evaluate the spatial distribution of *T. odontotermis* strains in termites' guts. The results suggested that *T. odontotermis* may be a metabolic symbiont located in the paunch compartment of the termite gut. Comparative genomics and analyses of carbohydrate-active enzymes indicated that it may aid in carbohydrate and amino acid metabolism, while an active Type VI Secretion System, may be helping *T. odontotermis* to overcome bacterial competition (Sapountzis, Gruntjes, et al. 2015).

The obligate fungiculture of the FG termites poses a challenge to the insects; plant material is highly carbon-rich relative to nitrogen, making the colony nitrogen limited. Termites are known to possess a great diversity of gastro-intestinal symbionts, and many studies have investigated how termites might make use of these symbionts to counteract the C/N imbalance created by a cellulose-based diet. The presence of nitrogen-fixing bacteria has been demonstrated in all families of termites (Sapountzis et al. 2016); however, nitrogen-fixation rates vary considerably inter- and intra-specifically and seem to be linked to host lifestyle. The importance of N-fixing bacteria to the nitrogen economy of fungus-growing termites has remained unresolved. In this project under my supervision, MSc student Jane de Verges investigated whether bacteria isolated from fungus-growing termites have the ability to fix nitrogen by screening them on N-free media in aerobic

and anaerobic conditions. Promising bacterial isolates were identified to the genus level through 16S rRNA sequencing and evaluated for potential N-fixation using real-time PCR amplification of the *nifH* gene. The functional acetylene reduction assay, which is the classic test for nitrogen fixation, showed positive AR activity in termite colonies of two FG termite species; *M. natalensis* and *O. badius*. In *M. natalensis*, the highest AR activity was in major workers, minor workers and minor soldiers, while major soldiers showed lower activity and the fungus comb showed the lowest activity. In *O. badius*, the highest activity was found in workers and then in soldiers, while the fungus comb showed very low AR activity, overall suggesting that atmospheric nitrogen fixation, facilitated by bacterial symbionts, may be more important for termites than for their fungus cultivar (Sapountzis et al. 2016).

I was also involved in Rafael's Rodrigues da Costa PhD thesis by providing consultation, aiding in genomic and statistical analyses and contributing to one of the peer-reviewed articles that came out of his thesis. Rafael thesis focused on different physiological aspects of this symbiosis, in order to gain a better understanding of: (i) the enzymatic aspects of plant biomass decomposition within the nest of fungus-growing termites; (ii) how the functional roles of gut symbionts reflect the fungal diet adopted by these termites; and (iii) how the growth capacity of Termitomyces fungi could explain the degree of interaction specificity displayed between the termite host and the fungus symbiont. I was mainly involved in the first part of his thesis, which characterized enzymes that are active during the decomposition process of plant material (which nutrients are ultimately used by the fungus). This part of his thesis showed that a diversity of active enzymes working synergistically at different stages of decomposition are likely responsible for a consistent decrease in plant components that is observed during the decomposition process (da Costa et al. 2017).

Finally, I also contributed to a study that was part of the PhD thesis of Saria Otani and showed that the composition of bacterial communities in the guts of FG termites is dependent on the host species, diet, and geographic location (Otani et al. 2018).

Non gut microbiota projects

Since 2013, I have also contributed to a number of projects that are the result of past collaborations initiated when I was a postdoctoral researcher at CSE. These projects were not directly related to the investigation of gut microbiomes of FG ants or termites, and my contribution was mostly related to microbiological methods, NGS data analysis, comparative genomics and microbial symbioses expertise. The bullet points below summarize these projects and publications produced:

- > I have contributed to an invited perspective paper on gut microbiomes of social insects (Sinotte et al. 2020). In that perspective article we discuss how division of labor in social insect colonies may have led to specialized microbiomes which could maximize the mutualistic benefits symbionts offer to their host.
- > I have been involved in a couple of projects related to the antibiotic producing cuticular Actinomycetes of FG ants. In 2012-2013 I helped to verify the findings of a 16S metagenomic study using PCR cloning and Sanger sequencing. This study (Andersen et al. 2013) showed that the *Actinomyces* communities that colonize the ant workers cuticle are highly stable and colony specific, where typically only a single Actinomyces strain is present, allowing the ants to reap the benefits of growing monocultures (Frank 1996). I am also currently contributing to another 16S metagenomic study that started in 2015 and the manuscript in preparation is expected to be submitted within 2023. In this project, I helped with the

investigation of the origins and specificity of antibiotic producing *Actinomycetes*. Our study showed that phylogenetically basal ant lineages (least advanced ant species) have Pseudonocardia gut symbionts that disappeared when related Pseudonocardia strains appeared on the cuticle of the higher attine ants (most evolved ant species). This suggests interaction specificity throughout, but with different forms of co-adaptation.

- In another joint 16S metagenomic project that we started with Associate Professor Rachelle Adams (OSU, USA) and is the continuation of an earlier study that started when we were both postdoctoral researchers in Copenhagen (Liberti et al. 2015), we have investigated the dynamics of transmission of different bacterial symbionts in fungus-growing ants (manuscript in preparation; Sadowski et al - to be submitted in 2022). During the earlier project, we co-supervised in the lab and the field MSc student Joanito Liberti who, as part of his MSc thesis, investigated the similarities between bacterial communities associated with FG ant species Social parasites (ants) often co-inhabit *Sericomyrmex amabilis* ant colonies and thus exploit the host resources. During this project, I was responsible for the microbiological and molecular part and Rachelle was responsible for the ecological part. During our ongoing project, which is spearheaded by Rachelle Adams' MSc student Victoria Sadowski, we have examined the bacterial community composition of infrabuccal pellets: pieces of fungus garden that incipient ant queens carry with them during their mating flight and therefore represent a mean to vertical transmission of microbes that are present in the fungus garden.
- Finally, I have also contributed to a study investigating the transmission of leafcutter ant gut bacteria (Zhukova et al. 2017). In this manuscript, which characterized the microbiota during ant development, I was responsible for helping and contributing to the amplicon sequencing analyses.

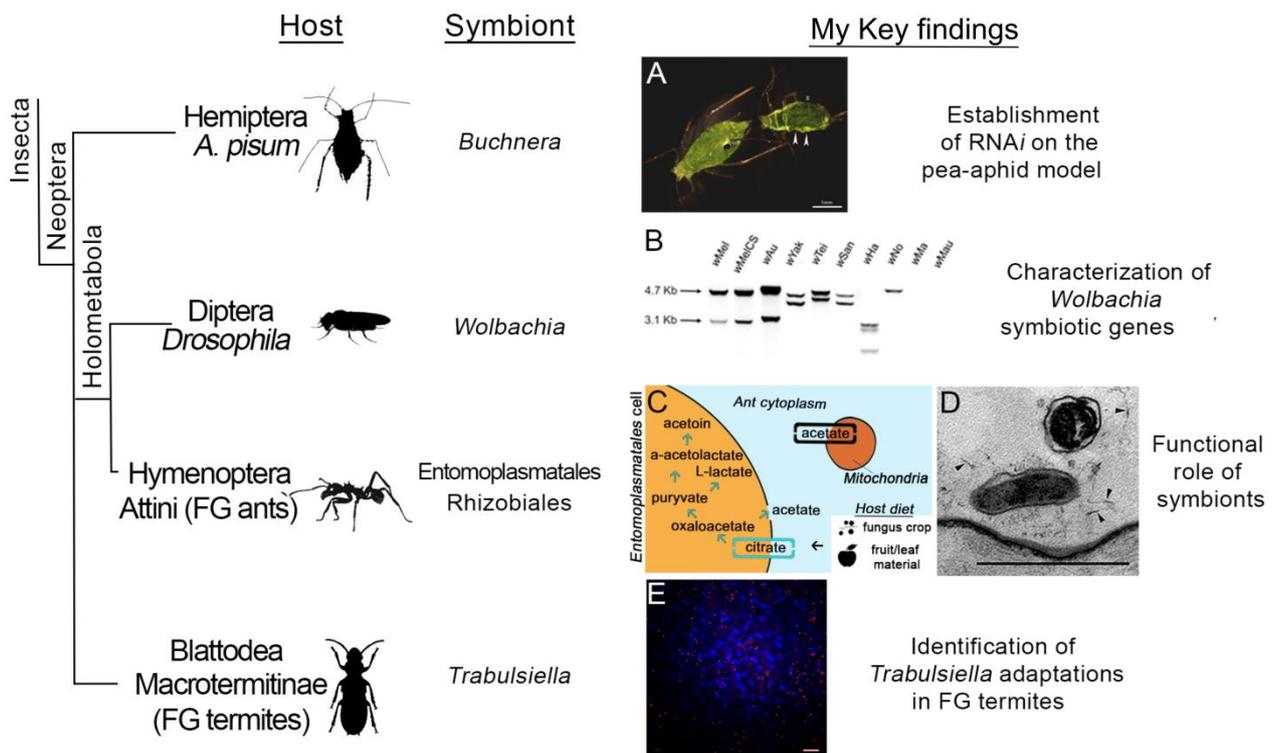


Figure 2: Summary of my past research in insect-bacterial symbioses. Insects hosts (left) have been grouped based on their taxonomy (www.ncbi.nlm.nih.gov/taxonomy), while next to them, the bacterial symbionts I investigated

(genus when available or else order) are presented. **A:** The establishment of the RNAi technique in aphids showed that *cathepsin-L* is involved in the aphid development, as its inactivation caused morphological deformities (arrowheads). **B:** Presence of bacteriophage genes in *Wolbachia* genomes (southern blot analysis). **C:** In leafcutter ants, Mollicutes bacteria import citrate (present in the host diet) and export acetate, which is being used by the ants' mitochondria. **D:** Electron microscopy image showing Rhizobiales bacteria surrounded by a low-density matrix (biofilm) in the ileum and rectum of leafcutter ants' guts. **E:** FISH confocal microscopy image of *Trabulsiella* bacteria (bright red dots) in the gut lumen of FG termites (blue color shows termite nuclei). For more details, see text and B1 part. All images were either obtained from phylopic (<http://phylopic.org/>), available under a Public Domain License, or adapted from my publications (Saridaki et al. 2011; Sapountzis et al. 2014; Sapountzis, Zhukova, et al. 2015; Sapountzis et al. 2018; Sapountzis, Gruntjes, et al. 2015).

Integration to MEDIS and overarching goals of my research program (2019 - now)

1. Background

There are approximately 700,000 strains of *Escherichia coli*, which are differentiated by a complex combination of surface, flagellar and capsular antigens (Segura 2018). Some of them have acquired virulence factors and are responsible for extra-intestinal pathologies such as UPEC (uropathogenic *E. coli*), NMEC (*E. coli* responsible for neonatal meningitis), and extra-intestinal *E. coli* (ExPEC). Other intestinal infections are DEC (Diarrhegenic *E. coli*). They are listed in 8 major pathotypes: enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), diffusely adherent *E. coli* (DAEC), shiga toxin *E. coli* or STEC (including EHEC), adherent invasive *E. coli* (AIEC), and recently shigatoxinogenic enteroaggregative *E. coli* (EAggSTEC) (Barbau-Piednoir et al. 2018). These pathotypes share virulence factors such as the tir-intimin system common to EPEC and STEC but also possess virulence factors which are specific to them such as adhesion factors (Diffusely adherent adhesin (DaaD) of DAEC), invasion (invasion plasmid antigen (ipa) of EIEC), toxins (Shiga toxin (stx) of STEC; enterotoxins of ETEC) or activating factors (Transcript activator adhesion fimbriae of EAggEC).

One of the most dangerous pathotypes, whose primary reservoir is cattle (Figure 3), is the Shiga toxin producing *Escherichia coli* (STEC) (Koutsoumanis et al. 2020; Sapountzis et al. 2020). STEC serotypes are defined by their ability to cause enteric disease, mainly through meat and dairy contamination, with symptoms that range from mild gastroenteritis to hemorrhagic colitis and Hemolytic-Uremic Syndrome (HUS). More than 400 STEC serotypes have been characterized and more than half of them have been associated with serious enteric disease (Brugère et al. 2012). Even though the annual number of cases is not as high as other zoonotic pathogens (Heredia and García 2018), STEC strains are extremely dangerous as their genome plasticity mediates the constant emergence of new potentially highly aggressive strains and treatment options are often limited (Koutsoumanis et al. 2020; Sapountzis et al. 2020). The serotype O157:H7, which is possibly the most well studied, has already resulted in epidemics in France, the US, and Japan (Brugère et al. 2012; Ferens and Hovde 2011), and is estimated to be responsible for approximately 405 million USD loss in the US every year (Fatima and Aziz 2019). Other important non-O157 outbreak agents include the *E. coli* O26:H11, O145:H28, O103:H2, O111:H8 (Elder and Nightingale 2013), and O104:H4, which affected more than 3500 and killed 53 people in 2011 in Germany (Burger 2012).

STEC (and often other Diarrhegenic *Escherichia coli*) carriage in cattle is asymptomatic, which makes it impossible to identify the host carriers in farms, while various methods for STEC control have been proposed with mixed results (Berry and Wells 2010). Treatment with probiotics, even though it has shown potential (Bertin et al. 2017; Wisener et al. 2015), can also harm native microbiota members, as direct-fed-microbials modes of action are not always specific (Bertin et al. 2017). Also, even though research on cattle to meat and dairy transmission has been extensive, we know relatively little about the origin and transmission of STEC in cattle farms and animals.

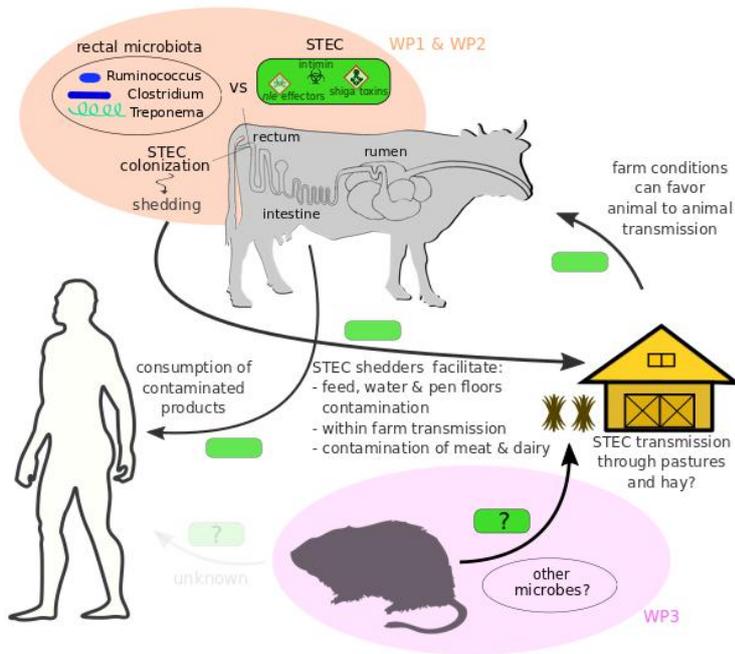


Figure 3: Overview of STEC and STEC flow (black arrows) in cattle farms. STEC's main reservoir is cattle (light gray). Even though STEC (illustrated by green squares) does not restrict to one GIT compartment, proliferation takes place exclusively in the rectum, which houses a diverse microbiota (top left; three of the most common/abundant genera are presented and shapes illustrate their morphology). STEC colonization can (but not always) result in STEC shedding, which facilitates transmission within farms and contamination of dairy products, which puts human health at risk: STEC virulence factors are presented inside

the illustrated green cell on top. The European water vole (bottom; dark gray) has been implicated as an STEC transmission vector. Silhouette images were modified from phylopic (phylopic.org), available under a Public Domain License.

Wild rodents - for example the European water vole (*Arvicola amphibius*) - are suspects for transmitting STEC (Gelling et al. 2012; Kilonzo et al. 2013), as previous studies have demonstrated that water voles can carry identical STEC strains to the ones in dairy cows (Nielsen et al. 2004) and the pathogen persists in rodent feces (Cízek, Literák, and Scheer 2000), which are often deposited in pastures where cattle graze, or the grass used for hay stock or silage (Figure 3). Similarly, blowflies (*Neomyia* sp.) and potentially houseflies (*Musca domestica*) in cattle farms are known pathogen vectors (Berry and Wells 2010; Puri-Giri et al. 2017), but studies that have investigated the carriage of dangerous pathotypes have been limited to the positive identification of virulence genes (Alam and Zurek 2004; Puri-Giri et al. 2017).

As far as understanding the persistence of EHEC/STEC and other potentially dangerous pathotypes in cattle, various studies have highlighted the importance of STEC interactions with the native microbiota in model systems (e.g. mice) for its persistence and survivability (Lustri, Sperandio, and Moreira 2017) and descriptive metagenomics in cattle have suggested potential interactions between STEC and native microbiota (Xu et al. 2014; Stenkamp-Strahm et al. 2018; Zaheer et al. 2017; Wang et al. 2018). These results however are not surprising as they simply confirm what we already know from other examples of animal symbioses (incl. humans); **through competitive or collaborative interactions with the microbiome and the host, invading pathogens and other microbes that enter the gastrointestinal niche persist or are quickly eliminated.** This is also why young infants are always the most susceptible; simply because their gastrointestinal tract has not been fortified by a diverse microbiota that typically covers the spatial and functional niches of the GIT. Thus, allowing less space for opportunistic pathogens and zoonotic threats to colonize and persist. Even though research in this direction is still in its infancy, it has great potential, especially when considering that the characterization of these interactions can provide the mean for the discovery of a new generation of Direct fed Microbials (DFM). DFM can potentially outcompete undesired

dangerous pathotypes in the GIT of domesticated animals and therefore offer a great alternative to antibiotics.

MEDIS research focuses on the study of the gastrointestinal tract of humans and animals and it is spread across three thematic categories: 1) the characterization of gut microbiota of humans and animals, the metabolic interplay between host and microbiota, the etiological agents of dysbiosis and interactions between host and gut microbiota, 2) the study of zoonotic food pathogens as well as host and microbial factors that favor or inhibit their transmission, and 3) the development of new galenic formulations with targeted activity. The overarching goal of this type of research is to improve the well-being and health of humans and animals and prevent (extra-) digestive diseases. The focus of my research falls under the second thematic category and more specifically STEC related research. MEDIS members have already published pioneering work that characterized the preferred STEC substrates (Bertin et al. 2013; 2014; 2018), factors that are crucial for successful STEC colonization (Monteiro et al. 2016), potential STEC antagonists that can be used as DFMs to prevent STEC colonization (Bertin et al. 2017), and the metabolic potential and functional roles of microbial symbionts in the cattle GIT (Comtet-Marre et al. 2017).

With my arrival at MEDIS in 2019, I complement the ongoing research by using whole-genome shotgun NGS, in-depth genomics, and (in the not-so-distant future) experimental evolution approaches that can examine the entire microbiota and how it interacts with STEC and other DEC. Such approaches will allow us to identify novel members of the ruminant gut microbiota that can prevent the colonization of pathogenic *E. coli*, but also the underlying genes and mechanisms that augment or reduce rates of evolutionary change in directions favorable or inhibitory for pathogens and zoonotes colonization. Except for a few studies from UMR454 Medis, which have explored the degradation potential of the gut bacterial communities of ruminants (Comtet-Marre et al. 2017) and the genomic potential of STEC, related to the pathogen's persistence, dissemination and infectivity (Segura, Bertoni, et al. 2018; Segura, Auffret, et al. 2018), such approaches appear to be largely lacking and I am confident I can help to develop them because of my dual training as an evolutionary and applied microbiologist and my experience in developing interdisciplinary microbiome-related research (as I did in my latest assignment as Senior Postdoc).

The three main objectives of my proposed research in my current position are to investigate:

- i) The impact of DEC on community composition and function of the cattle rectal microbiome using shotgun metagenomics on fecal samples from farms or artificial inoculation experiments.
- ii) The prevalence, co-occurrence or mutual exclusion between STEC and other microorganisms in the GIT and compare the identified patterns to similar ones in other STEC vectors such as rats, water voles, and blowflies.
- iii) The direction of evolutionary change in DEC and native microorganisms of the cattle GIT that are relevant to DEC colonization as a result of their interactions or pressure caused by environmental or dietary changes.

2. Projects

2.1 A global characterization of the fecal microbiomes of domesticated cattle: I initiated this study as soon as I arrived in Clermont Ferrand in the fall of 2019. I started by establishing a bioinformatic pipeline for the analysis of shotgun metagenomic data using data from a pilot study (Salaheen et al. 2018; 2019). This pipeline can: a) handle raw shotgun metagenomic data from cattle feces; from filtering until contig assembly, b) annotate the contigs to produce predicted ORFs and functionally annotate them to predict primary and secondary metabolites produced (using already published databases eggNOG, KEGG), c) bin the contigs based on similarities to available databases and sequence characteristics to create a representative set of Metagenome Assembled Genomes (MAGs), d) examine MAGs and contigs for their metabolic potential (through the presence of full KEGG metabolic pathways), virulence and antimicrobial resistance related genes, and e) use the contig coverage to assess the MAG abundance across samples. I have optimized the above pipeline for use with the local supercomputer at UCA (mesocentre.uca.fr), which allows the processing of large datasets in a few days.

My first research project (materialized into a manuscript, which is currently under review; Teseo et al., Microbiome) presents the first global gene catalog of cattle fecal microbiomes, a proxy of large intestine microbiomes, from 436 metagenomes from six countries. Seventy of these samples were collected from a local farm in Auvergne, while the rest came from earlier studies that used shotgun metagenomics to characterize the antimicrobial resistance potential of fecal microbiomes (Noyes et al. 2016; Zaheer et al. 2018; Rovira et al. 2019; Doster et al. 2018; Salaheen et al. 2019; 2020; Weinroth et al. 2019; Haley et al. 2020; Lim et al. 2020; Duarte et al. 2021). The advantage of using a large number of samples to produce individual assemblies is the large number (>30k) of MAGs (which after filtering was reduced to 2114 MAGs of >85% completeness and <5% contamination) that appeared to cover up to 70% of the entire bacterial diversity in feces. Phylogenomics suggested that the reconstructed genomes and their close relatives form distinct branches produce clustering patterns reminiscent of the metagenomics sample origin. Bacterial taxa had distinct metabolic profiles across classes and their complete metabolic pathways were mainly related to carbohydrate and amino acid metabolism. Dietary changes affected the community composition, diversity and potential virulence. Yet, predicted enzymes related to complete metabolic pathways remained present, albeit encoded by different microbes.

The findings of this study provide a global insight into the phylogenetic relationships and the metabolic potential of a rich yet understudied bacterial community (since studies typically focus on the rumen) and suggest that it can provide valuable services to the host. However, unlike the examples of invertebrate symbiosis, the members of this community are not irreplaceable. In line with previous findings, we tentatively confirm the hypothesis that symbionts of complex bacterial communities of mammals are expendable if there are substitutes that can perform the same task; i.e. seasonal dietary (and potentially other) changes can lead to a loss of diversity, which is likely countered by environmental microbes that can perform similar tasks for the host.

2.2 Contribution to (or continuation of) already ongoing projects in MEDIS

Since September 2019 I have been involved in:

1) A 16S metagenomic study that was carried out in young bulls in French cattle farms with the purpose of comparing the rectal microbial communities (collected feces) of animals that do or do not carry EHEC O157:H7. My contribution to this project was related to the statistical analyses and specifically the differential representation of bacterial OTUs between groups of animals with and without EHEC (Bibbal et al. 2022).

2) In 2016, the MEDIS group in collaboration with UMR IRSD Toulouse performed an artificial infection experiment during which a rifampicin resistant O157:H7 strain (originally isolated from a cattle farm) was administered to five young Holstein cows; five other cows of the same age and race acted as a control (NaCl administration). The experiment lasted three weeks, during which STEC-shedding was monitored (STEC was enumerated by culture on selective media) and feces were collected before and after the administration on day one, seven, fifteen and twenty (50 fecal samples in total). The advantage of this type of experiment is that it minimizes host variation which can often mask differences between treatment groups. I performed the bioinformatic and statistical analyses which identified various bacterial taxa with a significant preference towards STEC shedders or non-shedders: members of Ruminococcaceae and unclassified *Clostridiales* taxa were significantly reduced and members of Christensenellaceae and Lachnospiraceae families were significantly increased in STEC shedders compared to the control group, and these patterns were not present in the animals before the administration (manuscript in preparation; Bibbal et al.). I further plan to continue the above project by performing shotgun metagenomic sequencing on these samples, which will allow the functional characterization of the rectal microbial communities of dairy cows with and without STEC.

3) A meta-analysis led by ex-PhD student Valentin Ageorges investigated the phylogenetic distribution of the antigen 43 (Ag43) protein domains in all known complete *Escherichia* genomes. I have contributed to the bioinformatic and statistical analysis and the writing of the manuscript. The manuscript will be submitted by the end of 2022 (Ageorges et al.,)

2.3 Collaborations

France:

Dr. Anaïs Bompard is a young CRCN who was recruited in 2019 and joined the EPIA Unit in the INRAE center in Theix. Her skills in ecological modeling allow the building of dynamic (e.g. Generalized Lotka–Volterra) models of the ecological network that can be used to make predictions about the impact of perturbations and potential probiotic (or other types of) treatments. Therefore, her expertise complements mine perfectly in data analyses of metagenomic samples as my statistical skills focus on the identification and evaluation of statically significant contrasts and patterns while hers on prediction.

Dr. Celine Delbés (project TANDEM) is spearheading a joint project (TANDEM) that aims to assess the impact of changes in agricultural practices associated with the transition to agroecology on the functioning of the dairy system through a multidisciplinary approach focused on distinct (but intertwined) microbial compartments. This ambitious project is bringing together an interdisciplinary consortium of experts in different ecosystems to study holobionts relevant for the dairy system. The project aims to understand the overall functioning of grass-fed dairy farming systems, and the role of microbes in the meat and dairy production.

The overarching goals of this project are to:

- 1 Characterize the flow (nature, origin, intensity, and direction) of microorganisms (commensal and pathogenic) and genes (AMR, virulence, biomarkers) at different points and throughout the dairy system. Using global (shotgun) and targeted (qPCR) approaches, test the hypothesis of a « temporally oriented » causal chain that would connect the different compartments of the system via structural (strains) and/or functional (genes) microbial markers.
- 2 Evaluate the impact of changes in practices associated with an agroecological approach on the microbial communities (complexity, stability or resilience), and the change of flow in the system. The impact of these changes in the practices of the structure of microbiota will be assessed using a metabarcoding-type approach, which will be supplemented by an approach targeting the genes of interest (see above). Biotic and abiotic (physicochemical, biochemical) factors will be examined.

I will use my expertise in comparative metagenomics and multivariate statistics to identify and characterize the pathobiome and resistome (virulence and antimicrobial resistance genes) that are present in animal feces. Furthermore, I will use my expertise to identify significant contrasts between treatments, seasonal variation among collection dates and variation across animals and their compartments.

Dr. Delphine Bibbal & Dr Frédéric Auvray (ENVY-IRSD) have been working with DR Evelyne Forano for several years on projects related to STEC carriage by cattle. Our collaboration started with my involvement in an article that focused on the identification of factors related to STEC carriage in commercial farms in France (Bibbal et al. 2022). We intend to continue this collaboration by sequencing the 50 aforementioned fecal samples of the artificial infection experiment (see above). In addition, we submitted a proposal last year (as part of a joint submission involving multiple labs spearheaded by Dr. Sabrina Raynaud; INSTITUT CARNOT FRANCE FUTUR ÉLEVAGE (F2E) AAP 2021), which unfortunately was not funded and we resubmitted it this year (2022).

International:

The Fecobiome Initiative: Along with several other world renowned experts on cattle microbiome research, last year (2021) we started the fecobiome initiative. This international initiative aims at promoting research related to the cattle intestinal microbiota and at facilitating collaboration on relevant research projects by disseminating research results and increasing the public availability of resources. The overarching goals of this initiative are to ultimately help mitigate foodborne and animal pathogens that threaten livestock and human health, reduce the emergence and spread of antimicrobial resistance in cattle and their proximate environment, and potentially improve the welfare and nutrition of animals. In practice, the fecobiome initiative will exponentially increase the availability of samples, cultured microbial strains, and metagenomic data from other countries and will allow for relevant comparisons that can permit the evaluation of geographic location, diets, animal husbandry practices, temperature, season, animal race, etc. I started the discussion for the creation of this initiative through mail exchanges with Frank Moller Aarestrup at DTU (Denmark), Jo Ann Van Kessel at USDA (US), Garrett Suen at University of Wisconsin (US), George Tsiamis at the University of Patras and Professor Sharon Huws at the Queens University in Belfast (Ireland). This led to a perspective paper that we published last year (Sapountzis et al. 2021) and at the moment we are 17 researchers from eleven countries involved (fecobiome.com). Within its framework, I plan to start an ambitious cross-country program that will examine the genomic potential of thousands of microbial isolates coming from cattle farms across the world (see below).

Professor Frank Møller Aarestrup is the director of the Genomic Epidemiology group at the National Food Institute in Denmark. His group has collected hundreds of cattle fecal samples and isolated thousands of *Escherichia* strains from farms across Europe (primarily Denmark, but also France, Germany and the Netherlands). I met with him in August 2020 and he has agreed to collaborate as he is interested in the fecal microbiomes of cattle. He has already offered to finance shotgun metagenomic sequencing of fecal samples and to share available resources that include collected samples, metagenomic data, and microbial isolates from animal farms.

Dr. Serafino Teseo and Associate Professor Ajai Vyas: I met Associate Professor Ajai Vyas through an old collaborator (Serafino Teseo) who was at the time a postdoctoral researcher in the Ajai Vyas group. They have an interest in research related to houseflies and blowflies that may carry STEC and other DEC and they have financed the shotgun metagenomic sequencing of thirty samples coming from blowflies and co-financed the cattle fecal samples (collected in our experimental farm in Theix; ca. 70% of the cost was covered by them). In return, Dr. Serafino Teseo came to Clermont-Ferrand for one month (March/April of 2022) as part of his training in NGS and comparative genomic analyses.

For our current collaborative project with Dr. Teseo, we have used shotgun metagenomic approaches to characterize the microbes present in the blowflies' guts (*Neomyia cornicina*) collected in a cattle farm in Auvergne. The objective is to compare them to metagenomic data coming from cattle feces collected from the same farm to identify microbes that may be able to survive, transmit and proliferate in flies and the cattle GIT. Following the identification of these taxa we will focus on identifying genes (metabolic or other) that may allow these microbes to survive in multiple niches and may or may not be specific to the blowflies guts and the cattle feces.

Dr. Jo Ann Van Kessel is a research scientist in the Environmental Microbial & Food Safety Lab at the USDA. She and her team have had an interest in cattle rectal microbiota as a means to prevent pathogenic EHEC O157:H7 carriage (Salaheen et al. 2018; 2019). Even though they are not working anymore on STEC, they still have resources (data, bacterial isolates) that could be relevant to us and that they have agreed to share with us (personal communication). We will mainly collaborate through the Fecobiome initiative.

Dr. Philipp Engel is an Associate Professor in the Department of Fundamental Microbiology at the University of Lausanne. He and his team have significant expertise in bacterial comparative genomics. We are currently discussing the possibility of submitting a bilateral Swiss-French ANR grant.

Professor Stefano Morabito is the director of the Department of Veterinary Public Health and Food Safety of the Istituto Superiore di Sanità in Rome, Italy. I met director Stefano Morabito when he visited MEDIS and he has agreed to give us access to the institute's resources and share expertise regarding zoonotic diseases. I aim to collaborate with him in the preparation of a Horizon grant (within the next 5 years).

Professor Sharon Huws coordinates the global 'Rumen Microbial Genomics' network and is possibly the most renowned expert in rumen microbiota. She contributed to the writing of the Fecobiome initiative opinion paper and she has committed to help by contributing a substantial number of microbial isolates that will help kickstart a joint proposal for our initiative.

Associate Professor Garrett Suen: I have been in contact with Associate Professor Garrett Suen at the Department of Bacteriology, at the University of Wisconsin-Madison since early 2020. Dr. Garrett Suen is primarily interested in understanding i) the ecology and evolution of ruminal communities of cattle and how they may affect milk production, and ii) how rumen microbiota becomes established in developing calves, and in particular, how diet influences microbial composition. He and his team are also interested in the transmission of zoonotic pathogens in cattle farms and their survivability in pens, as well as their genome changes regarding antibiotic resistance genes. Dr. Garrett Suen is involved in the fecobiome initiative, but we are also discussing a joint project, during which we will artificially induce rumen acidosis in lactating dairy cows to identify biomarkers (present in the mouth and fecal microbiota) that will permit the identification of early signs of rumen acidosis. This experiment would involve me, Garret and his PhD student (Joe Skarlupka) who would come for a few weeks to visit and members of the Lallemand research team that is part of MEDIS (<https://www6.clermont.inrae.fr/medis/RECHERCHE/Les-projets-et-partenariat/Accueil-de-personnel-permanent-issu-de-societes-privees/SOCIETE-LALLEMAND>). My primary interest in this experiment is the emergence of acid-resistant virulence pathotypes and the changes in the occurrence and frequency of these pathotypes to animals that were affected by or resistant to rumen acidosis.

Associate Professor Eduardo Codo: I contacted Associate Professor Eduardo Codo in the summer of 2021 as he had performed an experiment during which he administered a STEC strain to young cattle. Even though Eduardo had no longer samples from the artificial inoculation experiment, he has been working with various bacterial and other pathogens and we currently collaborate on a new project that will examine the impact of *Cryptosporidium parvum* infection in young cattle. During the course of the experiment, Eduardo and his team administered *C. parvum* to young cattle and collected rumen, fecal, blood and other samples and at the moment we are five labs involved, each one examining the impact of

the *C. parvum* introduction to the biology of the animals. Using my expertise, I have examined the changes in bacterial pathogenic gene diversity as a result of the *C. parvum* introduction, reconstruct and examine MAGs coming from the data and examined how the artificial inoculation affected the entire community structure and composition. Preliminary results show that *C. parvum* infection causes a notable increase in *Clostridium* pathotypes (manuscript in preparation).

Professor Christian Menge: Professor Menge is the head of the institute of molecular pathogenesis at the Friedrich Loeffler Institute. Professor Menge is interested in the emergence and pathogenicity of virulent *Escherichia* (and other) pathotypes in livestock. A few years ago, he and his collaborators performed a large-scale inoculation experiment using a cocktail of different *Escherichia* pathogenic/zoonotic strains. We discussed the possibility of performing shotgun metagenomic sequences and comparative metaproteomic work in fecal samples from the artificially inoculated animals. In addition, we are currently preparing a joint DFG-ANR grant that we will submit in 2023 and will examine biotic and abiotic factors affecting the transmission of microbial strains in cattle farms.

2.4 Supervision of undergraduate students

Alice Leboucher (2021): Using an experimental farm in Theix, which is part of the INRAE facilities, fecal samples were collected across different seasons to examine changes in the microbiota as a result of seasonal and dietary changes. The purpose of these collections helped the initial project that I started when I arrived at the unit (2.1; Teseo et al., Microbiome under review), but also allowed for the collection of diverse microbial isolates (cultivated under aerobic conditions). Alice's internship aimed to establish a pipeline to quickly and efficiently isolate and characterize (using 16S rDNA) fecal strains. Using fecal material from three seasons (summer 2020, winter 2021, spring 2021), cultivable bacteria were isolated, grown in class-specific media (i.e. Enterobacteriae or non-Enterobacteriae specific) and 130 isolates were characterized to the level of species using 16S rDNA Sanger sequencing. The pipeline appears to be working successfully and all identified isolates (ca twelve genera) were part of the Proteobacteria and Firmicutes phyla.

Lucie Levet (2022): Lucie's internship partly continued Alice's project from last year but additionally examined an entirely new and understudied domain: the characterization of virulence genes of zoonotic/pathogenic bacteria in cattle feces using PCR based approaches in total DNA from fecal samples. Lucie's internship is divided into three parts: 1) evaluate known PCR primers that can target all known DEC, *Campylobacter* and *Listeria* pathotypes using previously characterized pathogenic isolates as control and DNA extracted from cow feces (that contains these virulence genes; verified by shotgun metagenomic sequencing), 2) design and evaluate new PCR primers based on the results of the first part to complement and (if necessary improve) the diagnostic potential of the primers and 3) examine the feasibility of all PCR primer pairs in quantitative PCR. Lucie's internship will help establish a pipeline for the quick identification and characterization of fecal (or other) DNA samples and microbial isolates that may carry virulence genes. Such a pipeline can provide a quick and cost-effective approach, especially for large projects during which we may need to examine hundreds or even thousands of samples for the presence of potential zoonotic pathotypes.

3. Perspectives (2023-...)

3.1 Identifying the network of interactions between microbiome members

Following the identification of putative symbionts in the cow lower intestine I plan to focus on the microbial interactions between members of the community and potential zoonotic/pathogenic bacteria that are occasionally present.

To identify these interactions and consequently potential antagonists of zoonotic and other pathogens in the cattle intestine, I plan to re-use the data from the functional characterization of the microbiome of feces (Teseo et al., in review) and the datasets from the artificial inoculation experiment of collaborator Christian Menge. Using the data from the artificial inoculation experiment, I will initially examine the cooccurrence and mutual exclusion of bacterial MAGs using standard methods (i.e. Spearman correlation; Quinn et al. 2017) and then I will enlist the help of collaborator Anais Bompard to develop Generalized Lotka-Volterra models (GLV) to describe the ecological interactions structuring the microbiome with and without the focal *Escherichia* pathotypes. Analytic and numeric explorations of this model will help evaluate the best levers of control of pathotypes within the microbial network. These results will help determine the relevant species to take into account and investigate the impact of our levers of control on ecosystem functions and interactions with the host. Following the predictions of this first part, I will then examine the cooccurrence of the identified MAGs with pathotypes in field samples (i.e. samples that were collected from various farms; dataset from Teseo et al., Microbiome submitted) and evaluate whether the identified mutual exclusion patterns hold. The third part of the bioinformatic analysis will examine the microbial metabolism of the identified antagonists and pathotypes.

Following the in-silico predictions and using qPCR and FISH microscopy, I plan to identify the site(s) in the rectum where potential bacterial interactions may take place. To achieve that I aim to use the intestines of young, slaughtered bulls from French slaughterhouses in the area of Auvergne. This project is still in development as I am currently discussing with stakeholders under what conditions we would be able to access these samples. If successful, this next round of experiments will allow us to confirm the previous findings and narrow down the list of potential defensive DEC antagonists. Subsequently, this will pave the way for functional *in vitro* competition experiments in mixed *in vitro* cultures. Facilities for *in vitro* cultures are already in place in MEDIS (biolevel safety 3 laboratories). The cultures will be used to perform competition assays that will examine the hypotheses formulated from the metagenomics part.

Having identified defensive symbionts that can inhibit STEC, their tissue localization and the primary and secondary metabolites they can produce, we should be able to pinpoint the key metabolites that have important roles in the interactions (i.e. competition) between the defensive symbionts and STEC. One such metabolite (the amino acid aspartate) has already been identified as a key metabolite that favors STEC colonization (Bertin et al. 2018). For this part, will use the *in vitro* mixed cultures mimicking the conditions of the cattle lower GIT, which we would have established in the previous part. Using a cutting-edge method that combines isotope probing and -omics approaches (meta-Stable Isotope Probing; (von Bergen et al. 2013), we could use labeled (e.g. ¹³C, ¹⁵N) substrates (e.g. aspartate and other potential key metabolites identified above), which would be added to mixed cultures. This project will be facilitated by the previously collected bacterial isolates and the international network of collaborators I will have created by then. The metabolites

would be catabolized and integrated into nucleic and amino acids by the bacteria. Then, we would extract DNA and proteins and those labeled with stable isotopes could be separated using methods similar to isopycnic centrifugation, which would allow us to acquire metagenomes/metaproteomes of only the organisms actively catabolizing the substrates of interest and their related metabolic products. This program would bring quantitative insights about: i) the efficiency of the defensive symbionts in outcompeting STEC in the presence of different key metabolites and the impact of these metabolites; and ii) the identification of the modes of action (i.e. better adapted metabolic pathways, secondary metabolites) used by the defensive symbionts. I have already explored the possibility of a collaboration with Professor von Bergen (UFZ, Germany), who pioneered this method, to evaluate its feasibility. *In vitro* cultures would be incubated in MEDIS, while the DNA and protein extraction, as well as the sequencing, would take place in his facilities. Alternatively (if this approach proves to be more troublesome than expected), I plan to use a local platform (DR Michel Hebraud at MEDIS is responsible for the proteomics part) which will identify and characterize proteins and metabolites: PFEM (<https://www6.ara.inrae.fr/medis/UMR-MEDiS/Expertises-et-competences-techniques/PFEM-Platform>).

3.2 Horizontal Transmission of microbes between cattle and insect vectors

Another direction that I plan to take my research is towards the characterization of the microbial flow from and to farm animals (which also includes potential bacterial pathogens). I will focus on:

- characterizing the microbial flow between farm animals and unexplored animal vectors and
- identifying the proximate and evolutionary mechanism that aid the dissemination, survivability, and ultimately adaptation of microbes in new niches.

One of these vectors, which I recently started working on in a project with collaborator S. Teseo, is the common blowflies (*Neomiya* species), which are almost always present in cattle pens (when temperatures > 15-20C). The lifestyle of these flies, their wide distribution and genome sequence (the genome of *Musca domestica* has already been sequenced (Scott et al. 2014) suggest that are capable of harboring a high bacterial diversity (Bahrndorff et al. 2017; Gupta et al. 2012; Scott et al. 2014) , which includes several animal and zoonotic pathogens like the enterohaemorrhagic *E. coli* (Alam and Zurek 2004; Wasala et al. 2013; Puri-Giri et al. 2017; Pava-Ripoll et al. 2015). Because of their vector potential, houseflies and blowflies are considered the most important non-biting pest (West 1951). Therefore, research towards this direction can provide insights about how to prevent the flies from acquiring pathogens and potentially limit pathogen transmission among and within farms.

Following the characterization of the microbial flow, I plan to ask for the funding of a follow-up project that will: 1) allow me to establish blowflies in the lab and use a model system to examine the microbial flow in targeted *in vitro* experiments, 2) cover the genome sequencing of the blowflies (only the *Musca domestica* genome has been sequenced and *Musca* flies are not the primary vector in French farms), 3) set up a large exploratory experiment in the field that will integrate macroecological, microbiological, genomic and metagenomic aspects to evaluate the potential of the microbial flow spread in the environment.

3.3 Explorative experimental evolution approaches

Using experimental evolution approaches, I plan to dissect the mechanisms that are responsible for genomic adaptations of:

- i) key members of the gut microbiota to retain beneficial interactions within the ruminant hosts and complete the nutrition or increase the productivity of the host or compete with invading pathogens, and
- ii) pathogenic DEC towards the evolution of higher (or lower) virulence traits.

The advantage of using such approaches is that by inducing genomic changes through artificial selection pressure *in vitro*, we can link internal (host) or external factors (diet, environment) to specific evolutionary mechanisms shaping bacterial genomes. For the DEC-microbiota interactions, the relevant selection pressure would be the bacterial competition between identified defensive symbionts and DEC, and therefore, I plan to use artificially constructed bacterial communities that would (or not; control experiments) be challenged with DEC. Furthermore, I would use different combinations of substrates to grow these bacterial communities to increase/decrease the pressure toward DEC (e.g. aspartate and other metabolites that will be identified by the previously proposed projects). For this project, I plan to use mixed *in vitro* cultures and potentially blowflies/houseflies as a bioreactor if by that time it is fairly straightforward to create wingless flies (possibly using CRISPR). The idea behind using the blowflies as a bioreactor is that the big microbial diversity they harbor and the increased tolerance as a result of their scavenging lifestyle suggests that competition among different bacterial species must be commonplace, and these flies would thus be an excellent model for studying cooperation and conflict within diverse bacterial communities (and relevant mechanisms) that can favor or inhibit pathogenic DEC.

This is the least developed plan and it will not start soon as it requires significant insight into the bacterial communities' interactions, but it is also the highest risk/most rewarding part of my proposed research.

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Selected publications

The Enterobacterium *Trabulsiella odontotermitis* Presents Novel Adaptations Related to Its Association with Fungus-Growing Termites

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Fungus-growing termites rely on symbiotic microorganisms to help break down plant material and to obtain nutrients. Their fungal cultivar, *Termitomyces*, is the main plant degrader and food source for the termites, while gut bacteria complement *Termitomyces* in the degradation of foodstuffs, fixation of nitrogen, and metabolism of amino acids and sugars. Due to the community complexity and because these typically anaerobic bacteria can rarely be cultured, little is known about the physiological capabilities of individual bacterial members of the gut communities and their associations with the termite host. The bacterium *Trabulsiella odontotermitis* is associated with fungus-growing termites, but this genus is generally understudied, with only two described species. Taking diverse approaches, we obtained a solid phylogenetic placement of *T. odontotermitis* among the *Enterobacteriaceae*, investigated the physiology and enzymatic profiles of *T. odontotermitis* isolates, determined the localization of the bacterium in the termite gut, compared draft genomes of two *T. odontotermitis* isolates to those of their close relatives, and examined the expression of genes relevant to host colonization and putative symbiont functions. Our findings support the hypothesis that *T. odontotermitis* is a facultative symbiont mainly located in the paunch compartment of the gut, with possible roles in carbohydrate metabolism and aflatoxin degradation, while displaying adaptations to association with the termite host, such as expressing genes for a type VI secretion system which has been demonstrated to assist bacterial competition, colonization, and survival within hosts.

Termites have a major influence on their ecosystems by their soil turnover processes and through their contributions to biodegradation of plant biomass (1). Approximately 30 million years ago, the termite subfamily Macrotermitinae developed fungus farming, and members of this subfamily can consume more than 90% of dry wood litter in tropical ecosystems (2). Fungus-growing termites have coevolved with a genus of basidiomycete fungi, *Termitomyces*, which is maintained in fungus combs within colonies, where it serves to decompose plant biomass (3) and as a food source for the termites (4). This ancient obligatory mutualism had a single origin in the African rainforest (5), and 11 genera and 330 species of fungus-growing termites have been described (6). The Macrotermitinae-*Termitomyces* symbiosis is found only in the Old World, with the highest level of diversity seen in Africa, where all termite genera are present, while only four termite genera are found in Asia (5).

Specific gut and fungus comb bacterial symbionts are associated with fungus-growing termites (7, 8; S. Otani, L. H. Hansen, S. J. Sørensen, and M. Poulsen, submitted for publication). While the importance of comb-residing symbionts remains largely unknown, the termites lose viability if the gut microbes are removed (9). The microbial gut community is highly complex, with ca. 150 phylotypes present (7, 8, 10). Gut communities are dominated by members of the *Bacteroidetes*, *Spirochaetes*, *Proteobacteria*, and *Firmicutes*, with *Alistipes* (*Bacteroidetes*), *Treponema* (*Spirochaetes*), and *Desulfovibrio* (*Proteobacteria*) generally being the most abundant (8, 9). The vast majority of these bacteria are unculturable, making their individual metabolisms and functional roles in the association largely unknown (1, 10, 11).

Two recent metagenomic analyses demonstrated that fungus-growing termites are able to degrade diverse plant substrates by

using enzymes derived from the fungal symbiont and from gut bacteria (12, 13). Gut bacteria in *Macrotermes natalensis* (12) and *Odontotermes yunnanensis* (13) termites contribute glycoside hydrolases (GHs) that complement those of *Termitomyces* in plant decomposition and also aid in termite digestion. Bacterial contributions are likely to be most important during the second of two gut passages of the plant substrate material: the first gut passage of the crude plant substrate and fungal asexual spores inoculates the substrate, which is subsequently decomposed by *Termitomyces* in the fungus comb (fungus garden), while the second gut passage of old comb ensures digestion of any remaining plant components and of fungal material (14). Although recent studies have identified certain gut bacterial community roles, we have limited insight

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into the specific pathways and metabolisms of individual gut members.

In 2007, Chou et al. isolated and characterized *Trabulsiella odontotermis* from the gut of the fungus-growing termite *Odontotermes formosanus* (15). This bacterium is a member of the *Gammaproteobacteria* of the family *Enterobacteriaceae*. The genus contains only one other described species, *Trabulsiella guamensis*, which was isolated from vacuum cleaner dust, soil, and human stool (16). Apart from phenotypic characterizations by Chou et al. (15), no work has explored the potential roles of *T. odontotermis* in the termite symbiosis. Using morphological identification of *T. odontotermis* from a large number of bacterial isolates, followed by 16S rRNA gene sequencing of candidate strains, we obtained nine *T. odontotermis* isolates from three termite genera. These strains were compared enzymatically to the type strains of *T. odontotermis* and *T. guamensis* by using API assays, their locations in worker and soldier guts were determined using fluorescence *in situ* hybridization (FISH), and insights into their potential functions and adaptations as gut symbionts were inferred from comparative draft genome analyses of two *T. odontotermis* strains, one *T. guamensis* strain, and a set of closely related members of the *Enterobacteriaceae*. Furthermore, we used genome information to design primers to investigate the distribution of *T. odontotermis* in different castes of 26 fungus-growing termite colonies and to determine whether putatively symbiosis-relevant *T. odontotermis* genes were expressed in termite guts.

MATERIALS AND METHODS

Isolates and morphological characterization of strains obtained from termite colonies. Two *T. odontotermis* isolates (Mn101-3w2C and Mn107-5a2C) were obtained in 2010 from microbial isolations from *Macrotermes natalensis* fungus-growing termites. Subsequent targeted microbial isolations from worker and soldier washes and gut contents, as well as freshly deposited and older fungus comb material, were performed during fieldwork collections in South Africa in 2011 and 2013. Isolations were performed by grinding biological material in phosphate-buffered saline (PBS), after which suspensions were plated on chitin (4 g chitin, 0.7 g K₂HPO₄, 0.3 g KH₂PO₄, 0.5 g MgSO₄·5H₂O, 0.01 g FeSO₄·7H₂O, 0.001 g ZnSO₄, 0.001 g MnCl₂, and 20 g of agar per liter), carboxymethyl cellulose (CMC; 10 g CMC and 20 g agar per liter), or nitrogen-free (N-free; 1 g KH₂PO₄, 0.2 g MgSO₄, 1 g CaCO₃, 0.2 g NaCl, 0.1 g FeSO₄, 5 mg Na₂MoO₄, 10 g glucose, and 15 g agar per liter) medium. Once in pure culture, more than 250 isolates were stored in 10% glycerol at -20°C. All isolates were streaked onto yeast extract-malt extract-agar medium (YMEA; 4 g yeast extract, 10 g malt extract, 4 g D-glucose, and 20 g agar per liter) and were subsequently left at room temperature for 96 h. To identify *Trabulsiella* isolates, we compared all isolates macromorphologically and microscopically (magnification, ×40) to the two *T. odontotermis* strains isolated in 2010 and to *T. guamensis* (DSM 16940). These were rod shaped, in accordance with the observations of Chou et al. (15), who described *T. odontotermis* to be 0.5 to 0.7 μm in diameter and 1.0 to 1.5 μm long. The reference strains were inoculated in triplicate to ensure consistent morphology.

All bacterial strains that resembled *Trabulsiella* morphologically were classified using 16S rRNA gene-specific PCR, with each reaction mixture containing 10 μl of PCR mix (2× VWR Ready PCR mix [VWR, Radnor, PA]), 8 μl Milli-Q water, and 1 μl each of the primers 27F and 1492R (15). The PCR protocol was 5 min at 96°C followed by 34 cycles of 45 s at 94°C, 45 s at 56°C, and 90 s at 72°C, with a final extension step of 5 min at 72°C, based on a previously published method (17). PCR products with positive bands on a 1.5% agarose gel were purified using an Invisorb fragment cleanup kit by Strattec Molecular and subsequently directly sequenced at the Beijing Genomics Institute. Sequences were assembled and checked

manually using the CLC Main Workbench (<http://www.clcbio.com/>) and searched against the NCBI nr database by using BLASTn (18). In addition to the two isolates whose genomes were sequenced, seven *T. odontotermis* isolates were obtained from the 2011 isolations, and two were obtained from the 2013 isolations (Table 1).

Physiological characterization of *Trabulsiella*. Eleven *Trabulsiella* strains were included in two API screens to explore their metabolic capabilities, including seven strains from 2011, the two sequenced (draft genome) strains, Mn101-3w2C and Mn107-5a2C (see below), *T. guamensis* DSM 16940 (16), and *T. odontotermis* DSM 22557 (15). We included *T. odontotermis* DSM 22557 because all *T. odontotermis* strains obtained as part of our study were from *Macrotermes* or *Microtermes* colonies, whereas the type strain was isolated from *O. formosanus* (15).

API testing involves standardized identification and characterization methods for Gram-negative rod-shaped bacteria (bioMérieux Inc., Durham, NC), which we used to obtain a basic idea of the metabolic potential of *T. odontotermis* and to test for differences between *T. odontotermis* and *T. guamensis*. For API 20E, the bacteria were grown for 24 h at room temperature on YMEA plates, after which a 5-mm inoculation loop was dipped into the colony and added to 5 ml of 0.85% NaCl solution. Samples were inoculated according to the instructions in the user manual for the bioMérieux API 20E set and were incubated at 36°C for 22 h. The required reagents were added, and the reading table provided in the manual was used to determine whether a reaction was positive or negative.

API ZYM is a method for examining enzyme activities, and we hypothesized that enzymatic differences between the *T. odontotermis* and *T. guamensis* species could be linked to differences in symbiotic versus free-living lifestyles. Bacterial cultures were grown for 24 h at room temperature on YMEA plates, after which bacteria were added to 5 ml distilled water until the turbidity of the suspension reached a 5 to 6 McFarland standard (19). The API ZYM strip was inoculated according to the instructions in the user manual for the bioMérieux API ZYM set and was incubated for 4 h at 37°C. The final reagents were added, and the API ZYM reading table was used to score the enzymatic reactions.

FISH. Termites from an *M. natalensis* colony (Mn140) (Lajuma, 23°02.285'S, 29°26.441'E), an *Odontotermes* cf. *badius* (i.e., suspected to be *O. badius*) colony (Od126) (experimental farm, 25°43'55.7"S, 28°14'08.2"E), and an *Odontotermes* sp. colony (Od130) (Mookgophong, 24°40'30.5"S, 28°47'50.4"E) were collected in South Africa in 2013, and their guts were dissected, placed in 1× PBS in Eppendorf tubes, and stored at 4°C in 4% paraformaldehyde (PFA) in 1× PBS. Five guts were dissected from workers and soldiers of colonies Od126 and Od130, as well as five guts from major workers, minor workers, major soldiers, and minor soldiers from colony Mn140. The gut consists of the crop, the gizzard, the midgut, the paunch, the colon, and the rectum, and the Malpighian tubules are also connected to the gut. Only the gizzard, the midgut, the paunch, the colon, and the rectum were consistently intact, so these were investigated.

To permeabilize membranes, tissues were incubated at 60°C in 70% acetic acid for 1 min and then rinsed and incubated for 5 min in PBS at room temperature. The tissues were subsequently dehydrated through a graded ethanol series and left at room temperature for a few minutes to dry out. For deproteinization, tissues were incubated in 0.01 N hydrochloric acid with 0.1 mg/ml pepsin for 10 min at 37°C. After deproteinization, the tissues were rinsed, incubated for 5 min with PBS, and then dehydrated in ethanol and air dried as described above. The tissues were then prehybridized for 30 min at 45°C in a prehybridization buffer made with 79% hybridization buffer (0.9 M NaCl, 20 mM Tris, and 5 mM EDTA in water, pH 7.2), 20% Denhardt solution (5 g Ficoll, 5 g polyvinylpyrrolidone [PVP], and 5 g bovine serum albumin in 500 ml water), and 1% SDS. Hybridization was performed for 3 h at 45°C, using prehybridization buffer supplemented with 0.75 μg/μl of a *Trabulsiella*-specific probe, after which Alexa 647 red fluorescent dye was used for staining. The *Trabulsiella*-specific probe was obtained using Geneious 4.8.5 (31), and it tar-

TABLE 1 Identification of *Trabulsiella* isolates based on 16S rRNA gene sequencing

Isolate ID ^a	Termite origin	Yr of isolation	Location	<i>Trabulsiella</i> strain ^b	GenBank accession no.	% identity
Mn101-3w1C	<i>Macrotermes natalensis</i>	2011	Rietondale, 25°43'45.2"S, 28°14'05.8"E	<i>Trabulsiella odontotermitis</i> strain Eant 3-3	DQ453130.1	99
				<i>Trabulsiella odontotermitis</i> strain Eant 3-9	NR_043860.1	99
				<i>Trabulsiella</i> sp. Of17	AB673461.1	99
Mn105-2a1M	<i>Macrotermes natalensis</i>	2010	Mookgophong, 24°40'30.5"S, 28°47'50.4"E, 1,046 m	<i>Trabulsiella guamensis</i> NBRC 103172	AB681979.1	99
				<i>Trabulsiella guamensis</i> GTC 1379	AB273737.1	99
				<i>Trabulsiella odontotermitis</i> strain Eant 3-3	DQ453130.1	98
				<i>Trabulsiella guamensis</i> NBRC 103172	AB681979.1	98
Mn107-4a2C	<i>Macrotermes natalensis</i>	2010	Rietondale, 25°43'45.2"S, 28°14'05.8"E	<i>Trabulsiella guamensis</i> NBRC 103172	AB681979.1	98
				<i>Trabulsiella guamensis</i> GTC 1379	AB273737.1	98
				<i>Trabulsiella odontotermitis</i> strain Eant 3-3	DQ453130.1	98
				<i>Trabulsiella guamensis</i> NBRC 103172	AB681979.1	98
Mn101-W+p2M	<i>Macrotermes natalensis</i>	2011	Mookgophong, 24°40'30.5"S, 28°47'50.4"E	<i>Trabulsiella odontotermitis</i> strain Eant 3-3	DQ453130.1	99
				<i>Trabulsiella odontotermitis</i> strain Eant 3-9	NR_043860.1	99
				<i>Trabulsiella</i> sp. LB10	JQ864379.1	99
Mn105-3a4C	<i>Macrotermes natalensis</i>	2011	Mookgophong, 24°40'30.5"S, 28°47'50.4"E, 1,046 m	<i>Trabulsiella guamensis</i> NBRC 103172	AB681979.1	99
				<i>Trabulsiella guamensis</i> GTC 1379	AB273737.1	99
				<i>Trabulsiella odontotermitis</i> strain Eant 3-3	DQ453130.1	98
				<i>Trabulsiella guamensis</i> NBRC 103172	AB681979.1	98
Mn107-Yc2C	<i>Macrotermes natalensis</i>	2011	Experimental farm, 25°43'55.7"S, 28°14'08.2"E	<i>Trabulsiella guamensis</i> NBRC 103172	AB681979.1	98
				<i>Trabulsiella guamensis</i> GTC 1379	AB273737.1	98
				<i>Trabulsiella odontotermitis</i> strain Eant 3-3	DQ453130.1	98
				<i>Trabulsiella odontotermitis</i> strain Eant 3-9	NR_043860.1	99
Od126-sC	<i>Odontotermes badius</i>	2013	Rietondale, 25°43'45.2"S, 28°14'05.8"E	<i>Trabulsiella odontotermitis</i> strain Eant 3-3	DQ453130.1	99
				<i>Trabulsiella odontotermitis</i> strain Eant 3-9	NR_043860.1	99
				<i>Trabulsiella</i> sp. Of17	AB673461.1	99
Od126-wNF	<i>Odontotermes badius</i>	2013	Rietondale, 25°43'45.2"S, 28°14'05.8"E	<i>Trabulsiella odontotermitis</i> strain Eant 3-3	DQ453130.1	99
				<i>Trabulsiella odontotermitis</i> strain Eant 3-9	NR_043860.1	99
				<i>Trabulsiella</i> sp. Of17	AB673461.1	99
				<i>Trabulsiella odontotermitis</i> strain Eant 3-3	DQ453130.1	99
Mi101-Bc4C	<i>Microtermes</i> sp.	2011	Rietondale, 25°43'45.2"S, 28°14'05.8"E	<i>Trabulsiella odontotermitis</i> strain Eant 3-3	DQ453130.1	99
				<i>Trabulsiella odontotermitis</i> strain Eant 3-9	NR_043860.1	99
				<i>Trabulsiella</i> sp. Of17	AB673461.1	98

^a Strain IDs are as follows: Mn101-3w1C, *Macrotermes natalensis* colony 101 (Mn101), worker 3 (3), wash (w) isolate 1 on chitin medium (1C); Mn105-2a1M, *Macrotermes natalensis* colony 105 (Mn105), worker 2 (2), abdomen (a) isolate 1 on microcrystalline medium (1M); Mn107-4a2C, *Macrotermes natalensis* colony 107 (Mn107), worker 4 (4), abdomen (a) isolate 2 on chitin medium (2C); Mn101-w+p2M, *Macrotermes natalensis* colony 101 (Mn101), wash and gut content (w+p) isolate 2 on microcrystalline medium (2M); Mn105-3a4C, *Macrotermes natalensis* colony 105 (Mn105), worker 3 (3), abdomen (a) isolate 4 on chitin medium (4C); Mn107-Yc2C, *Macrotermes natalensis* colony 107 (Mn107), young comb (Yc) isolate 2 on chitin medium (2C); Od126-sC, *Odontotermes* cf. *badius* colony 126 (Od126), soldier, chitin (C); Od126-wNF, *Odontotermes* cf. *badius* colony 126 (Od126), worker, nitrogen-free medium (NF); Mi101-Bc4C, *Microtermes* sp. colony 101-B (Mi101B), comb material (c) isolate 4 (4) on chitin medium (C).

^b The top three hits are shown for each isolate.

geted a region within the *rhdD* gene (*Trabulsiella*-specific probe sequence, TACCAGGACAGCAGTATGATAAAGA).

Following hybridization, all samples were kept in the dark, and tissues were rinsed in hybridization buffer with 0.1% SDS twice at

45°C, followed by rinsing in PBS and subsequently in water. The slides were air dried and mounted with an aqueous mounting medium made for fluorescence (Gel Mount), with DAPI (4',6-diamidino-2-phenylindole) (VectaShield HL1200). Slides were kept in the dark at 4°C

TABLE 2 Genome statistics for the draft genomes of *T. odontotermis* and *T. guamensis*

Parameter	Value or description		
	<i>T. odontotermis</i> Mn101-3w2C	<i>T. odontotermis</i> Mn107-5a2C	<i>T. guamensis</i> DSM 16940
Genome sequencing method	Illumina paired-end sequencing	Illumina paired-end sequencing	Two Roche 454 libraries (8-kb paired-end library and unpaired shotgun library)
Genome size (bp)	4,819,652	4,786,520	4,929,751
No. of contigs	192	150	278
Minimum contig length (bp)	200	137	200
Mean contig length	25,090	33,654	17,733
N_{50}	79,484	108,358	73,732
Maximum contig length	189,137	243,230	214,713
% G+C	55.1	51.8	53.6
% coding regions	90	88	89
No. of ORFs	4,639	5,234	4,754
No. of rRNA genes	2	4	5
No. of tRNA genes	68	68	62

until observations using a Leica TCS SP2 laser scanning confocal microscope.

Genome sequencing of *T. guamensis* and *T. odontotermis*. The type strain of *T. guamensis* was purchased from ATCC (Manassas, VA), and DNA was extracted at the University of Wisconsin Genome Evolution Laboratory, after which the genome was sequenced as part of a project to increase the breadth of genome sequence data available for the *Enterobacteriaceae* (<http://atol.genetics.wisc.edu>).

For *T. odontotermis*, DNAs were extracted from two isolates (Mn107-5a2c and Mn101-3w2C) that were initially cultured on YMEA plates and subsequently cultured in Luria-Bertani (LB; 10 g tryptone, 5 g yeast extract, and 5 g NaCl per liter) broth. Cultures were centrifuged, the supernatants were removed, and DNAs were extracted using a Qiagen DNeasy blood and tissue kit (Qiagen, CA). Whole-genome sequencing was performed using a mate-paired Illumina HiSeq method with 500-bp inserts at the Beijing Genomics Institute. Sequence reads were assembled using Velvet (20). Sequencing and assembly metrics for the two genomes are shown in Table 2.

Gene annotation and genome comparisons. Open reading frames (ORFs) were predicted for each contig by using GeneMark (21) and were annotated using BASys to compare functional categories (22). KEGG analyses using the BLAST algorithm and single best hit (SBH) with default settings were performed to identify and compare pathways in *T. odontotermis*, *T. guamensis*, and the following set of closely related *Enterobacteriaceae*: *Citrobacter rodentium* ICC168, *Enterobacter cloacae* ATCC 13047, *Salmonella enterica* serovar Typhimurium SL1344, *Klebsiella pneumoniae* 342, and *Escherichia coli* 536 (see Table S1 in the supplemental material). We further explored the presence of carbohydrate-active enzymes (CAZymes) in *T. odontotermis*, *T. guamensis*, and their close relatives by using the CAZymes Analysis Toolkit (CAT) (23) (Table 3).

For nucleotide and amino acid sequence comparisons and to detect gene duplication events in *Trabulsiella*, we performed local BLAST searches (18) with a cutoff E value of $1e-50$ and a 50% identity threshold. For all hits, we checked the length of the query versus the subject to confirm that they were not gene fragments, and we considered only those with ORF identities of $>70\%$ to be paralogs. The predicted amino acid sequences of ORFs present more than once (possible paralogs) were compared among the *Trabulsiella* strains. ORFs present (or present multiple times) in *T. odontotermis* but not in *T. guamensis* are given in Tables S1 and S2 in the supplemental material. For proteins of particular interest, we performed a local BLAST search (E value cutoff of $1e-50$; 50% identity) against the Swiss-Prot database (24) and annotated them by using Pfam (25) and KEGG (26). The complete Pfam-A v.26.0 database (25) was retrieved locally (<ftp://ftp.sanger.ac.uk/pub/databases/Pfam/releases/Pfam26.0/Pfam-A.hmm.gz>) and formatted using the hmmpress command, and the *Trabulsiella* genomes were analyzed using the hmmscan

command in HMMER v.3.1b1 (27), with an E value cutoff of 10^{-2} and the `-cut_ga` option, which uses the “gathering threshold” set for each family in Pfam. Proteins present only in *Trabulsiella* were analyzed further by using the InterPro Web tool (<http://www.ebi.ac.uk/interpro/>) and BLAST searches against the NCBI nr database.

Phylogenetic analysis. The 16S rRNA genes from the nine isolates obtained from the 2011 and 2013 isolations were placed phylogenetically along with those of the *Trabulsiella* type strains and those of representative members of the *Enterobacteriaceae*, obtained from GenBank. Sequences were aligned using MEGA 5.2.2 (28), and bootstrap support was evaluated using maximum likelihood analyses under the HKY+G+I model of sequence evolution and neighbor-joining analyses, generated with 500 bootstraps and performed in MEGA. For multilocus sequence typing (MLST), the following 10 housekeeping genes were chosen, based on previous studies (29, 30): *atpD* (ATP synthase subunit beta), *dnaK* (chaperone protein), *infB* (translation initiation factor IF-2), *glnA* (glutamine synthetase), *gltA* (citrate synthase), *gyrB* (DNA gyrase subunit B), *pnp* (polyribonucleotide nucleotidyltransferase), *recA* (RecA protein), *rpoB* (DNA-directed RNA polymerase subunit beta), and *thrC* (threonine synthase). Genes were extracted from the *Trabulsiella* genomes by use of customized scripts and local BLAST searches, while closely related *Enterobacteriaceae* genomes were downloaded from the NCBI database, and genes were identified using BASys (22). Sequences were aligned using MEGA 5.10 (28), and a maximum likelihood phylogeny was generated with 1,000 bootstrap iterations (general time-reversible model with G+I rates).

Determining the presence of *Trabulsiella* in guts of diverse fungus-growing termites. To determine how frequently *T. odontotermis* is present in the guts of fungus-growing termites, we designed primers for *T. odontotermis*-specific genes (see Table S3 in the supplemental material) by using the Geneious software vR7 (31) and performed a PCR screen of 135 samples originating from 26 colonies of four fungus-growing termite species (three genera) from South Africa (see Table S4). Queen and king guts were dissected individually, while 10 termite guts per worker or soldier caste per colony were dissected and pooled. DNA extraction was performed using a Qiagen blood and tissue kit (Qiagen, Germany). DNA was eluted in 100 μ l AE elution buffer, and PCR conditions were as follows: denaturation for 3 min at 94°C followed by 35 cycles of 30 s at 94°C, 30 s at 58°C, and 45 s at 72°C and a 7-min final extension at 72°C. PCR products were visualized on a 1.5% agarose gel.

Reverse transcription-PCR (RT-PCR) confirmation of the expression of genes putatively relevant to symbiont function and symbiosis maintenance. Ten guts from major workers from two colonies of *M. natalensis* (Mn156 [experimental farm, 25°43'55.7"S, 28°14'08.2"E] and Mn162 [Mookgophong, 24°40'30.5"S, 28°47'50.4"E]), two colonies of *Odontotermes* sp. (Od128 and Od159 [experimental farm, 25°43'55.7"S,

TABLE 3 Carbohydrate-active enzyme (CAZyme) genes identified in the draft genomes of *T. odontotermis* and *T. guamensis* based on CAT (<http://mothra.ornl.gov/cgi-bin/cat/cat.cgi>) predictions

CAZyme family ^a	No. of genes in genome							
	<i>Trabulsiella guamensis</i> DSM 16940	<i>Trabulsiella odontotermis</i> Mn101-3w2C	<i>Trabulsiella odontotermis</i> Mn107-5a2C	<i>Escherichia coli</i> 536	<i>Enterobacter cloacae</i> subsp. <i>cloacae</i> ATCC 13047	<i>Citrobacter rodentium</i> ICC168	<i>Salmonella</i> Typhimurium strain SL1344	<i>Klebsiella pneumoniae</i> 342
GH1	4	5	4	4	6	2	2	10
GH2	2	2	2	3	2	1	0	2
GH3	4	4	5	2	3	3	2	3
GH4	2	2	2	2	3	3	2	5
GH5	0	0	0	0	1	1	0	0
GH8	1	1	1	1	1	1	1	2
GH9	0	0	0	0	0	0	0	1
GH13	6	8	6	7	11	10	9	9
GH18	3	2	2	1	2	8	0	0
GH19	1	1	1	0	2	0	0	1
GH20	0	0	0	0	1	0	0	0
GH23	21	20	20	5	11	9	8	8
GH24	0	0	0	0	5	2	4	2
GH28	0	0	0	0	0	0	0	1
GH30	0	0	0	0	0	0	1	0
GH31	2	1	1	1	3	3	3	2
GH32	0	0	0	2	1	0	0	2
GH33	0	0	0	1	1	1	1	0
GH36	0	0	0	0	2	0	0	1
GH37	2	2	3	2	2	2	2	2
GH38	0	0	0	0	1	0	0	0
GH39	0	0	0	0	0	0	0	1
GH42	0	0	0	0	1	0	0	2
GH43	2	3	2	0	2	1	0	3
GH53	0	0	0	0	1	0	0	1
GH63	0	0	0	1	0	0	0	0
GH65	1	1	1	1	1	1	0	0
GH73	2	2	2	2	3	1	2	1
GH77	1	1	1	1	1	1	1	1
GH78	0	0	0	0	0	0	0	1
GH88	2	2	2	0	1	2	0	0
GH94	0	0	0	0	0	0	0	1
GH102	1	1	1	1	1	1	1	1
GH103	1	1	1	1	1	1	1	1
GH104	0	0	0	0	0	0	0	1
GH105	0	0	0	0	1	2	1	2
GH108	3	0	0	0	0	3	1	0
GH109	5	9	8	0	0	0	0	0
GH127	0	2	1	0	1	2	1	0
GH NC	0	0	0	0	1	0	1	0
GT1	0	0	0	1	0	0	1	0
GT2	22	21	27	11	14	8	13	9
GT4	8	8	8	7	6	7	8	7
GT5	1	1	1	1	1	1	1	1
GT8	0	0	0	3	0	2	2	0
GT9	3	3	3	3	4	3	3	5
GT17	0	1	0	0	0	0	0	0
GT19	1	1	1	1	1	1	1	1
GT20	2	2	2	1	1	1	1	1
GT26	1	1	1	1	1	1	1	2
GT28	1	1	1	1	1	1	1	1
GT30	0	0	0	1	1	1	1	1
GT35	2	5	2	2	2	2	2	3
GT39	0	1	1	0	0	0	0	0
GT41	1	1	1	0	1	0	0	0
GT44	0	0	0	0	0	1	0	0
GT51	6	6	6	4	4	3	4	4

(Continued on following page)

TABLE 3 (Continued)

CAZyme family ^a	No. of genes in genome							
	<i>Trabulsiella guamensis</i> DSM 16940	<i>Trabulsiella odontotermis</i> Mn101-3w2C	<i>Trabulsiella odontotermis</i> Mn107-5a2C	<i>Escherichia coli</i> 536	<i>Enterobacter cloacae</i> subsp. <i>cloacae</i> ATCC 13047	<i>Citrobacter rodentium</i> ICC168	<i>Salmonella</i> Typhimurium strain SL1344	<i>Klebsiella pneumoniae</i> 342
GT52	0	0	0	0	2	0	0	0
GT56	1	1	1	1	1	1	1	1
GT60	0	0	0	0	0	1	0	0
GT73	0	0	0	0	0	1	1	1
GT83	0	0	0	1	1	0	1	1
GT84	0	0	0	0	0	0	0	1
GT NC	0	0	0	3	1	0	0	2
CE1	10	5	8	1	0	0	0	1
CE4	2	1	2	1	1	0	2	2
CE8	1	1	1	1	1	1	1	1
CE9	10	9	10	2	2	1	1	1
CE10	1	5	3	0	0	0	0	0
CE11	3	3	3	1	1	1	1	1
CE NC	0	0	0	3	1	1	3	1
PL5	1	1	1	0	0	0	0	0
PL22	0	0	0	0	0	1	0	1
CBM4	0	0	0	0	0	0	0	1
CBM5	0	0	0	5	3	9	1	0
CBM13	0	0	0	0	0	0	1	0
CBM34	0	0	0	1	1	1	1	1
CBM35	0	0	0	0	0	0	0	1
CBM41	0	0	0	0	0	0	0	1
CBM48	0	0	0	2	4	4	4	3
CBM50	2	2	2	2	5	2	5	5
Total	145	150	151	99	134	117	106	128

^a GH, glycoside hydrolases; GT, glycosyl transferases; CE, carbohydrate esterases; PL, polysaccharide lyases; CBM, carbohydrate-binding modules.

28°14'08.2"E]], and one colony of *Odonotermes* cf. *badius* (Od150 [Rietondale, 25°43'45.2"S, 28°14'05.8"E]) collected in 2015 were dissected and stored in RNAlater at -80°C. Total RNA was extracted using an RNeasy minikit (Qiagen, Germany). One microgram of total RNA was treated with RQ1 RNase-free DNase I (Promega Corporation, Madison, WI), and 900 ng of this product was reverse transcribed with an iScript RT kit (Bio-Rad) to obtain first-strand cDNA. As a negative control, the remainder of the DNase-treated RNA was examined by PCR under the same conditions. All *T. odontotermis* gene-specific PCRs were performed on cDNA, DNase-treated RNA, *T. odontotermis* DNA, and water. PCR conditions were as follows: denaturation for 3 min at 94°C followed by 40 cycles of 30 s at 94°C, 30 s at 58°C, and 45 s at 72°C and a 7-min final extension at 72°C. PCR products were visualized on a 1.5% agarose gel.

Nucleotide sequence accession numbers. Contigs for the draft genomes of *T. guamensis* ATCC 49490, *T. odontotermis* Mn107-5a2c, and *T. odontotermis* Mn101-3w2C have been deposited in GenBank under accession numbers [JMTB000000000](#), [JNGH000000000](#), and [JNGI000000000](#), respectively.

RESULTS

Distribution, phylogeny, and physiology of *T. odontotermis*.

We detected *T. odontotermis* in the guts of fungus-growing termites by using the following two independent methods. (i) We isolated *Trabulsiella* strains from workers of a total of 7 colonies (9 isolates) collected in 2011 and 2013 (Table 1) by using culture-based approaches. (ii) We PCR amplified *Trabulsiella*, using specific primers, from 5 of 11 *M. natalensis* colonies, 3 of 5 *Odonotermes* sp. colonies, 1 of 5 *Odonotermes* cf. *badius* colonies, and 3 of 5 *Microtermes* colonies (see Table S4 in the supplemental material). Collectively, the results of the combination of a culture-

dependent and a culture-independent approach support the hypothesis that *Trabulsiella* is a facultative symbiont frequently present in fungus-growing termite guts.

Trabulsiella is placed within the *Enterobacteriaceae*, but previous analysis did not provide sufficient resolution for a solid placement of *T. odontotermis* and *T. guamensis* in a global *Enterobacteriaceae* phylogeny (Fig. 1A). 16S rRNA gene-based phylogenetic placement of *Trabulsiella* confirmed its placement within the *Enterobacteriaceae* (Fig. 1B) and placed the two *T. odontotermis* isolates closest to each other, with *T. guamensis* being basal to both (Fig. 1B). MLST analysis of 10 housekeeping genes provided strong bootstrap support for the placement of both *T. odontotermis* and *T. guamensis* separate from the other *Enterobacteriaceae*.

Figure 2 provides the results of the API 20E and API ZYM tests, among which the API 20E test revealed identical results for all *T. odontotermis* strains, with a lack of H₂S production in *T. guamensis*. API ZYM testing showed only slight differences between the different *T. odontotermis* and *T. guamensis* scores, as they were primarily either all negative (scores of 1 and 2) or all positive (scores of 3 to 5) (Fig. 2).

FISH confocal microscopy of workers and soldiers of *Macrotermes* and *Odonotermes* colonies showed similar patterns, with *Trabulsiella* being present in highest abundance in the paunch and in the rectum and, to a lesser extent, in the colon (Fig. 3). While *Trabulsiella* was most abundant in the paunch, many other bacteria were abundant in the midgut and rectum (Fig. 3).

Genome statistics and *Trabulsiella* metabolism. The *T. odontotermis* genomes were approximately 4.8 Mb long, 68 tRNA

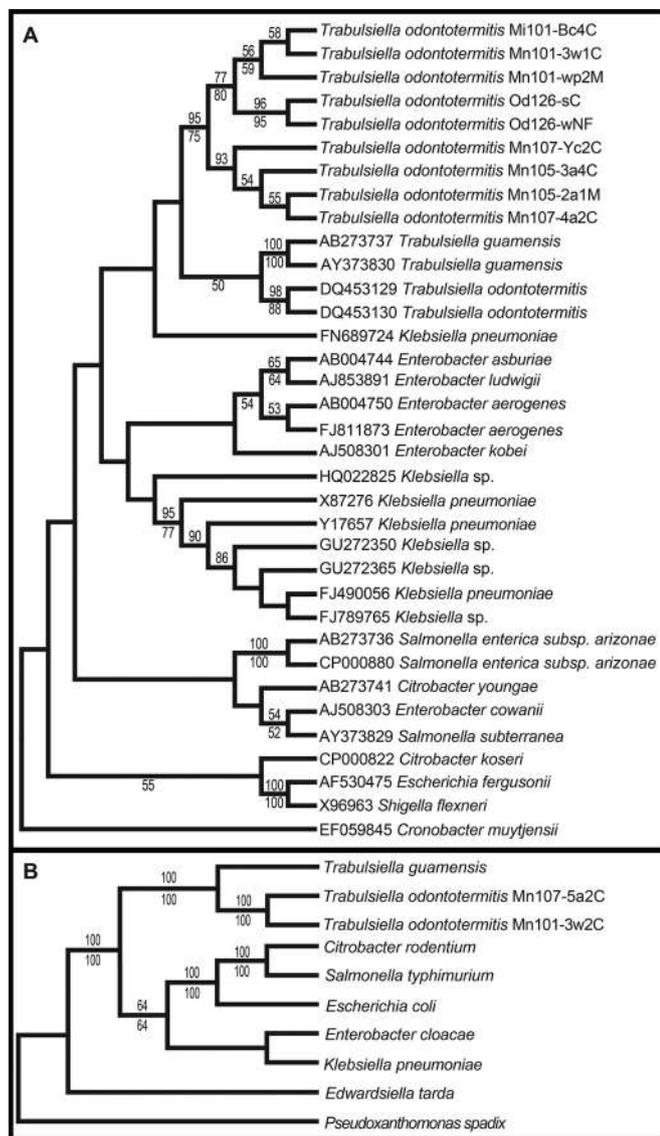


FIG 1 (A) 16S rRNA gene phylogram for all obtained *Trabulsiella odontotermis* isolates and closely related *Enterobacteriaceae*. Bootstrap support values for neighbor-joining (above branches) and maximum likelihood (below branches) conditions and 500 pseudoreplicates are given for nodes with support values of >50. (B) Phylogenetic tree based on MLST of 10 genes (see the text for details). Bootstrap support values for neighbor-joining (above branches) and maximum likelihood (below branches) conditions and 1,000 pseudoreplicates are given for nodes with support values of >50.

genes were identified in each genome, GC contents ranged from 51.8 to 55.1% for *T. odontotermis*, and the GC content was 53.6% for *T. guamensis* (Table 2). All three *Trabulsiella* strains have the genetic machinery for heterotrophic, aerobic metabolism and, like most close relatives, all genes for glycolysis, the tricarboxylic acid (TCA) cycle, the pentose phosphate pathway, and oxidative phosphorylation. Like their close relatives, *Trabulsiella* strains have the machinery for dissimilatory nitrate reduction (nitrate to nitrite to ammonia). However, *T. odontotermis* has duplications in all four *nar* genes (*narG*, *narH*, *narI*, and *narJ*), and these are organized as two separate gene clusters similar to those in other *Enterobacteriaceae*, but this appears not to be the case for *T. guamensis* (see Table S2 in the supplemental material).

Potential of *Trabulsiella* for substrate degradation and possible roles in termite symbiosis. Both *T. odontotermis* strains have more encoded CAZymes than *T. guamensis* and other closely related *Enterobacteriaceae* (Table 3). Among the glycoside hydrolases (GHs), the most abundant families are GH23, which includes peptidoglycan lytic transglycosylases and a chitinase, and GH109, with the only known activity being that of an α -*N*-acetylgalactosaminidase. The most abundant glycosyl transferase (GT) family is GT2, encoding numerous activities, including the biosynthesis of disaccharides, oligosaccharides, and polysaccharides. *T. odontotermis* Mn107-5a2C has more GT2 genes than *T. guamensis* and *T. odontotermis* Mn101-3w2C, but all three *Trabulsiella* genomes contain 2 to 3 times more GT2 genes than those of their close relatives (Table 3). All strains have carbohydrate esterases (CEs), most abundantly the CE1, CE9, and CE10 families, containing enzymes capable of the hydrolysis of carboxylic esters.

The comparative KEGG analysis also showed that *T. odontotermis* has genes related to degradation of various compounds, which in many cases are absent in its close relatives. These include a unique cytochrome P450 gene in the *T. odontotermis* strains, which is absent from *T. guamensis* and close relatives that have other P450 genes. *T. odontotermis* Mn101-3w2C also has a haloalkane dehalogenase, able to degrade dichloropropane and dichloroethane, which is absent in *T. guamensis* and also in other *Enterobacteriaceae*; a tautomerase, which could be involved in the degradation of xylene; and a choloylglycine hydrolase, catalyzing the hydrolysis of the amide bond in conjugated bile acid, present only in *T. odontotermis*, *Klebsiella*, and *Enterobacter* (see Table S1 in the supplemental material).

To better understand the potential roles of the above-mentioned enzymes, we investigated their positions in KEGG metabolic pathways by comparison of the KEGG annotations assigned to ORFs identified in *M. natalensis* gut metagenomes from workers, soldiers, and a queen (12). None of the enzymes in the metagenomic analysis were identical to *Trabulsiella* enzymes, but all metabolic pathways identified in worker and soldier metagenomes (bisphenol, chlorocyclohexane, chloroalkane, and dioxin degradation, xenobiotic degradation by cytochrome P450, bile acid, and tropane piperidine and pyridine alkaloid biosynthesis) were also present in *T. odontotermis*, suggesting conserved functions in the termite gut. Several genes encoding enzymes belonging to these pathways thus appear to be redundant with genes in the termite, the mutualistic *Termitomyces* fungus, or other gut bacteria. Expression analysis using *Trabulsiella* gene-specific primers and cDNAs obtained from RNAs extracted from *M. natalensis* and *Odontotermes* sp. worker guts showed that the genes encoding cytochrome P450, allose kinase, and NifA were transcribed (see Fig. S1 in the supplemental material).

Genes related to host colonization and bacterial competition. Comparing *T. odontotermis* to genomes from close relatives exposed several genes that could be involved in the invasion and colonization of the host niche and that may provide an advantage in microbial competition. The *Trabulsiella* genomes contain a gene encoding a β -lactamase, which could provide resistance to penicillin by turning it into inactive penicilloic acid, which has also been demonstrated for *T. odontotermis* *in vivo* (15). The xanthine dehydrogenase (XDH) identified in *T. odontotermis* Mn107-5a2c is critical for biofilm formation in *Enterococcus faeca-*

API 20E											
Enzymatic activity	Mn101-Bo4C	Mn107-Yc2C	Mn105-2a1M	Mn101-3w1C	Mn101-3w2C	Mn105-3a4C	Mn107-5a2C	Mn101-W+p2M	Mn107-4a2C	DSM-22557	<i>T. guamensis</i>
β-galactosidase											
Arginine dihydrolase											
Lysine decarboxylase											
Ornithine decarboxylase											
Citrate utilization											
H ₂ S production											
Urea hydrolysis											
Tryptophan deaminase											
Indole production											
Acetoin production											
Gelatinase											
Fermentation/oxidation glucose											
Fermentation/oxidation mannitol											
Fermentation/oxidation inositol											
Fermentation/oxidation sorbitol											
Fermentation/oxidation rhamnose											
Fermentation/oxidation sucrose											
Fermentation/oxidation melibiose											
Fermentation/oxidation amygdalin											
Fermentation/oxidation L-arabinose											
Oxidase											
NO ₃ ⁻ to NO ₂ ⁻ conversion											
NO ₃ ⁻ to N ₂ conversion											
Oxidation glucose											
Fermentation glucose											
API ZYM											
Control											
Alkaline phosphatase											
Esterase (C4)											
Esterase lipase (C8)											
Lipase (C14)											
Leucine arylamidase											
Valine arylamidase											
Cysteine arylamidase											
Trypsin											
α-chymotrypsin											
Acid phosphatase											
Naphthol-AS-BI-phosphohydrolase											
α-galactosidase											
β-galactosidase											
β-glucuronidase											
α-glucosidase											
β-glucosidase											
N-acetyl-β-glucosaminidase											
α-mannosidase											
α-fucosidase											

FIG 2 (Top) Results of API 20E enzyme activity assays for 11 *Trabulsiella* strains. Dark gray, positive result; light gray, negative result. Activity for citrate utilization (medium gray) was not clear. (Bottom) Results of API ZYM enzyme activity assays for 11 *Trabulsiella* strains. On the five-point scale, scores of 1 and 2 (two lightest gray shades) are considered negative, while scores of 3 to 5 are considered positive (three darkest gray shades), with increasing enzyme activity.

lis (32). The tetrathionate transporters found in *T. odontotermis* Mn107-5a2c appear to provide a growth advantage in *Salmonella* Typhimurium over competing microbes during gut inflammation (33, 34). Also, the spermidine putrescine transport system, duplicated in *T. odontotermis* (K11069 to K11071), plays a critical role in invasion and intracellular survival of *Salmonella* Typhimurium (35). However, for both the XDH and the tetrathionate transporters, no indications of active expression were found, suggesting that these genes are either tightly regulated or remnants of inactive genes (see Fig. S1).

A striking difference between the two *T. odontotermis* isolates and other *Enterobacteriaceae* (including *T. guamensis*) was the absence of type II, type III, and type IV secretion systems in *T. odontotermis*. However, all three *Trabulsiella* strains have type VI secretion system (T6SS) genes on three different loci, similar to *Erwinia* sp., *Pantoea* sp., and *Pseudomonas aeruginosa* (36, 37). Two of the three loci appear to be conserved in *Trabulsiella*, while one differs between *T. odontotermis* and *T. guamensis* (Fig. 4; see Table S5 in the supplemental material). Based on amino acid comparisons, all locus II and almost half of the locus III components (11/23 components) are more similar to T6SS components in *Yersinia* sp., while approximately half of the components of locus I (10/20 components in Mn101-3w2c and 13/20 components in Mn107-5a2c) are more similar to components in *S. enterica*, where they have been hypothesized to be essential for survival in and colonization of host cells (38). Expression analysis using *Trabulsiella* T6SS-specific primers showed that T6SS genes from all three loci are transcribed in *Odontotermes* species and from two out of three loci in *M. natalensis* (Fig. 4; see Fig. S1). More specifically, the three copies of *icmF* are transcribed from their respective loci, *impC* is expressed from both loci checked, and *impJ/vasE* is expressed from its single locus, while RT-PCR did not reveal detectable transcription for the *vasG/clpV* genes at both loci examined (Fig. 4).

DISCUSSION

***Trabulsiella odontotermis* is a putative facultative symbiont of fungus-growing termites.** Isolates of *T. odontotermis* were obtained across different termite species and genera: three different *M. natalensis* colonies, one *Microtermes* sp. colony, and two *Odontotermes* cf. *badius* colonies, including a previously described isolate (15). Multilocus sequence typing (Fig. 1B) allowed for solid placement of *T. odontotermis* as a separate species from *T. guamensis* and other members of the *Enterobacteriaceae*, providing better branch support than 16S rRNA gene analysis alone (Fig. 1A) (15), with bootstrap support of 100 for the node separating the *Trabulsiella* genus from other *Enterobacteriaceae* and 100 for the node before it (Fig. 1B).

It appears that *T. odontotermis* is often present in the fungus comb, from which it was also isolated, and in the paunch of the termite gut, where FISH revealed it to be abundant (Fig. 3). We did not identify *T. odontotermis* genes in a search against data from a recent metagenomic study (12), which could be the result of the high complexity of the bacterial communities in the fungus-growing termite gut or of an insufficient sequencing resolution to identify *T. odontotermis* and its genes, or *T. odontotermis* may simply not have been present in that specific colony. However, a PCR screen of 135 samples from three fungus-growing termite genera showed that *T. odontotermis* was present in workers and soldiers in 20% to 45% (depending on the fungus-growing ter-

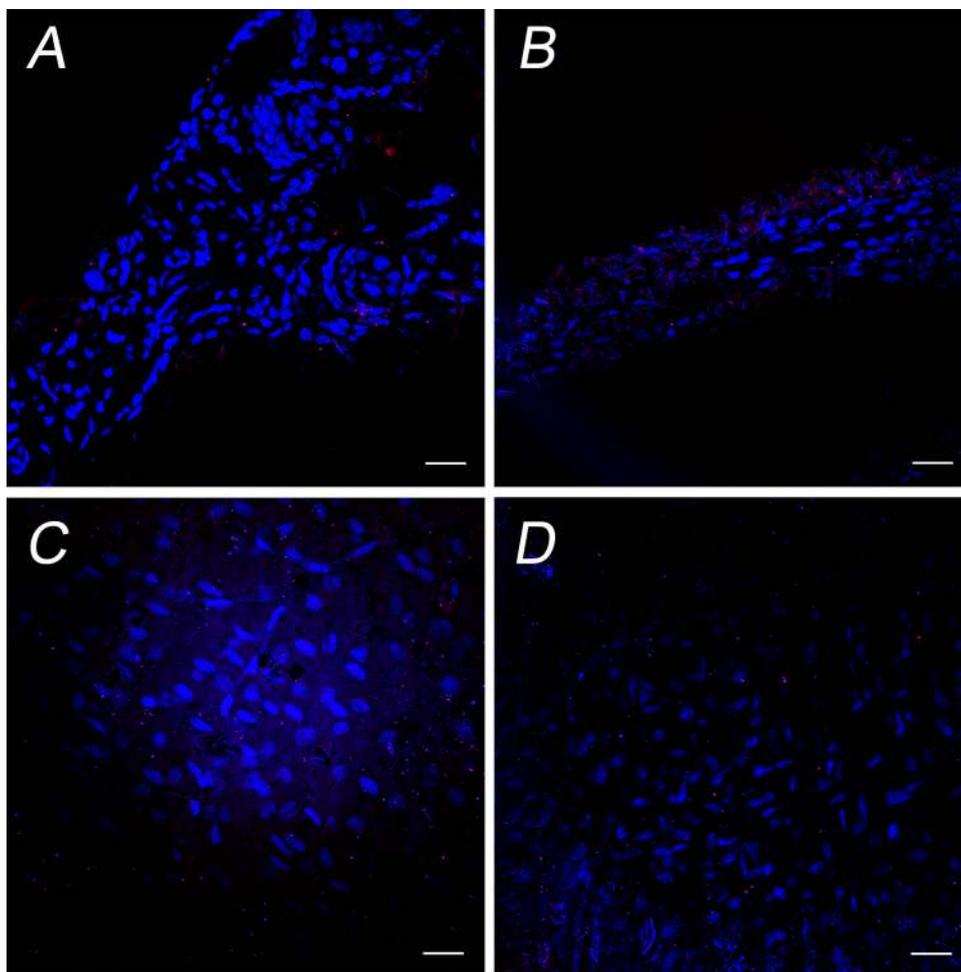


FIG 3 Representative laser scanning confocal microscopy images of various gut tissues of *Odontotermes* soldiers. (A) Colon; (B) midgut; (C) paunch; (D) rectum. *Trabulsiella* bacteria can be seen as bright red/pink spots, while other bacteria are seen as bright blue spots and the insect cell nuclei are blue. The faint diffuse cytoplasmic blue (B and C) and red (B) areas, observed mostly in pictures of the colon and the paunch, represent nonspecific staining. Bars, 20 μ m.

mite species) of all colonies tested (see Table S4 in the supplemental material).

***T. odontotermis* genome analyses suggest possible roles in degradation, nutrition, and protection against fungal toxins.** The presence of *T. odontotermis* in the paunch of its termite host suggests a role for the bacterium in termite metabolism. We tested the degradation capabilities of *T. odontotermis* by using API tests, which indeed showed activities of various enzymes involved in plant biomass degradation, such as β -galactosidase and α -glucosidase, indicating a potential role in carbohydrate metabolism for *T. odontotermis* in the symbiosis. CAZyme analysis also identified several enzymes (GH23, GH109, CE1, CE9, CE10, and GT2) that were more abundant in *Trabulsiella* than in other *Enterobacteriaceae* (Table 3). Most CAZyme families appeared to be expanded in *Trabulsiella* and had diverse functions, including functions in chitin metabolism (CE9 and GH23) (www.cazy.org). Chitin degradation was expected, as six of the nine *Trabulsiella* isolates were obtained using a chitin-based microbiological medium, and chitin metabolism may be essential for fungus-growing termite gut bacteria for the prevention of antagonistic fungi entering the nest (during the first gut passage of the plant substrate) or for the degradation of *Termitomyces* biomass for the acquisition

of nutrients (both gut passages). Genes for chitin breakdown could, however, also be involved in digestion of the chitin-containing termite peritrophic membrane (39). Thus, more explicit experimental testing of whether *Trabulsiella* is involved in plant and fungus degradation is warranted.

Although several genes appeared to be unique to the *T. odontotermis* genomes, including genes we expected could play a role in symbiotic *T. odontotermis*, only a few of these genes were transcribed, including a gene encoding a CYP450 enzyme which may play a role in the degradation/detoxification of the mycotoxin aflatoxin B1. It has been demonstrated that the plant damage caused by fungus-growing termites stimulates *Aspergillus* growth (40), suggesting that termites may unintentionally help *Aspergillus* to spread through plant material, at their potential detriment. Detoxification enzymes could counter the negative effects of aflatoxins, which could be important during the first gut passage. Aflatoxins can be toxic for animals, humans, and insects, including termites (41–43), and it has been demonstrated that CYP450, which is present and expressed in *T. odontotermis* in both *M. natalensis* and *Odontotermes* cf. *badius* guts (see Fig. S1 in the supplemental material), can metabolize and possibly counter the toxic effects of aflatoxin B1 (43–45).

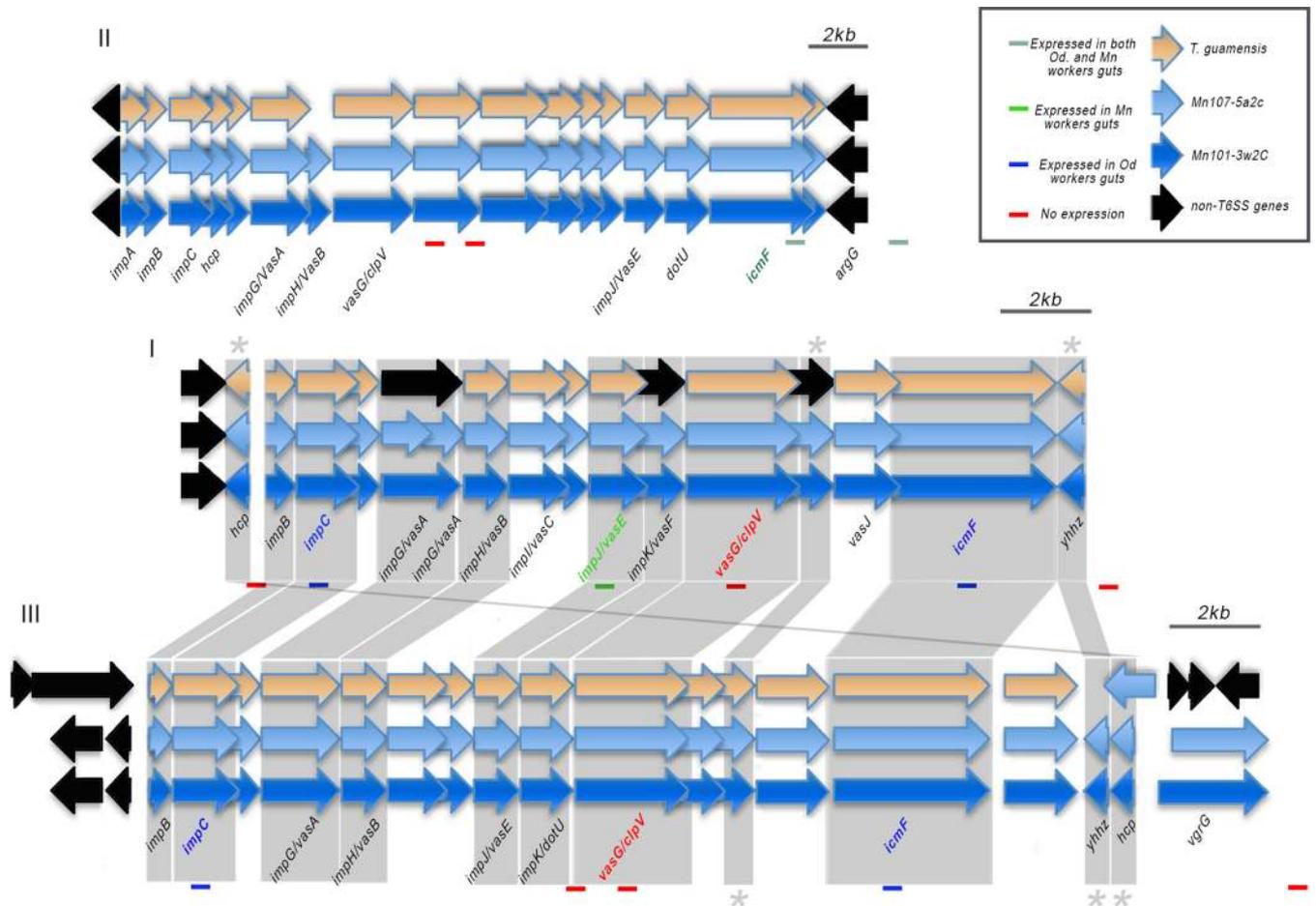


FIG 4 Schematic representations of the three different T6SS gene clusters in *T. odontotermis* Mn101-3w2C (dark blue), *T. odontotermis* Mn107-5a2c (light blue), and *T. guamensis* (brown). Arrows are proportional to the sizes (bp) of the genes in the three loci, and the orientations of genes are indicated. KEGG annotations of the predicted proteins are given below each arrow (ORF). Loci I and III have several genes with similarities in the amino acid sequences of their predicted proteins (E values of $>1e-50$; similarities of $>50\%$). All paralogs are highlighted and connected with gray shading. The gray asterisks show paralogs that exist in *T. odontotermis* but not in *T. guamensis*. For more details regarding all T6SS loci in all three *Trabulsiella* strains, see Table S5 in the supplemental material. Colored bars under the genes (arrows) show the gene expression results for *M. natalensis* (Mn) and *Odontotermes* sp. (Od) worker guts (red, no expression; green or blue, expression of the gene). A bar placed under the middle of a gene means that the primers used were specific only for that gene; a bar placed between two neighboring genes means that the primers were specific for two different neighboring genes and therefore amplified part of each gene and the region between them (in order to investigate whether they are expressed as an operon).

The ability to switch from aerobic to anaerobic conditions could be a general adaptation of fungus-growing termite symbionts. Based on the API tests, the enzymatic profiles of *T. odontotermis* were similar to those of *T. guamensis*, with the only observed difference being the ability of *T. odontotermis* to reduce sulfate. This dissimilarity is probably at the transcriptional level, since we did not observe genome differences. Many bacteria utilize sulfate as an electron acceptor to respire anaerobically to obtain energy by oxidizing organic compounds or molecular hydrogen while reducing sulfate to hydrogen sulfide (46); this process has been described for the gut of the termite *Mastotermes darwiniensis* (47). This difference may therefore reflect a necessary adaptation for *T. odontotermis* to be able to live in the paunch, which has been demonstrated to be anaerobic in wood-feeding higher termites (48–50).

It is common for bacteria to have the genetic machinery for both aerobic and anaerobic metabolism. *T. odontotermis* seems to have invested in nitrate reduction genes, as duplicated genes

exist for catabolizing nitrate and regulating this pathway (see Table S2 in the supplemental material). These adaptations could be related to *T. odontotermis* turning to anaerobic metabolism when necessary, potentially allowing for the bacterium to colonize both the fungus comb and the paunch. However, the termite gut may present a gradient from oxic to anoxic conditions (50), which would predict that such adaptations are necessary for gut symbionts. *T. odontotermis* metabolism may remain active in both gut and comb environments, which likely is also the case for other bacteria present in both these environments of the association (Otani et al., submitted). Little is known about the roles of most bacteria present in both guts and combs, but their ability to switch between environments may allow them to perform symbiont functions in both places to possibly allow for more efficient biomass breakdown, nutrient absorption, and/or detoxification at the termite colony level.

***Trabulsiella* genes putatively involved in symbiosis maintenance.** Genetic mechanisms for host association exist in both mu-

tualistic and pathogenic associations, where they facilitate successful colonization of the eukaryotic host, as theory suggests that symbiotic relationships evolve between competing strains, with more virulent strains outcompeting others. For example, mechanisms that govern this adaptability in the *Euprymna scolopes* squid-*Vibrio fischeri* mutualism include quorum sensing, two-component regulatory mechanisms, cell signaling, and differential adhesion to the host light organ. Furthermore, to successfully colonize the light organ, *V. fischeri* has to outcompete other bacteria in the early stages of the symbiosis (reviewed in reference 51).

The fungus-growing termite gut and fungus comb environments harbor complex bacterial communities (10; Otani et al., submitted), making it likely that associated symbionts would need to compete with other microorganisms for successful colonization of the host. *T. odontotermis* has several genes that are infrequent in other *Enterobacteriaceae* and several duplicated genes related to sensing environmental change, surviving host defenses, resistance, biofilm formation, and competition against other microbes (see Tables S1 to S3 in the supplemental material). Many of these genes could help *T. odontotermis* in competition with other bacteria. Among the most interesting are the genes for the T6SS, which is the only secretion system in *T. odontotermis*. Several T6SS genes are transcribed from all three loci (Fig. 4), including the *icmF* gene, which is believed to be a key virulence factor of closely related *Gammaproteobacteria* pathogens and is always present in all T6SSs examined (52). Studies on the T6SS in *Vibrio cholerae* have shown that it aids in bacterial competition through a sophisticated system that delivers toxic compounds, but it is able to discriminate between competitors and self (53, 54). *Vibrio* and *Pseudomonas* use this system to kill both prokaryotic and eukaryotic cells through delivery of toxic compounds (54, 55). Differences between *T. odontotermis* and its close relatives may reflect adaptations for symbiosis establishment and for competition with other microbes. Such traits are likely to be found not only in *T. odontotermis* but also in other gut bacteria, where they very well may be essential for survival.

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The evolution of abdominal microbiomes in fungus-growing ants

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Abstract

The attine ants are a monophyletic lineage that switched to fungus farming ca. 55–60 MYA. They have become a model for the study of complex symbioses after additional fungal and bacterial symbionts were discovered, but their abdominal endosymbiotic bacteria remain largely unknown. Here, we present a comparative microbiome analysis of endosymbiotic bacteria spanning the entire phylogenetic tree. We show that, across 17 representative sympatric species from eight genera sampled in Panama, abdominal microbiomes are dominated by Mollicutes, α - and γ -Proteobacteria, and Actinobacteria. Bacterial abundances increase from basal to crown branches in the phylogeny reflecting a shift towards putative specialized and abundant abdominal microbiota after the ants domesticated gongylidia-bearing cultivars, but before the origin of industrial-scale farming based on leaf-cutting herbivory. This transition coincided with the ancestral single colonization event of Central/North America ca. 20 MYA, documented in a recent phylogenomic study showing that almost the entire crown group of the higher attine ants, including the leaf-cutting ants, evolved there and not in South America. Several bacterial species are located in gut tissues or abdominal organs of the evolutionarily derived, but not the basal attine ants. The composition of abdominal microbiomes appears to be affected by the presence/absence of defensive antibiotic-producing actinobacterial biofilms on the worker ants' cuticle, but the significance of this association remains unclear. The patterns of diversity, abundance and sensitivity of the abdominal microbiomes that we obtained explore novel territory in the comparative analysis of attine fungus farming symbioses and raise new questions for further in-depth research.

KEYWORDS

16S-MiSeq sequencing, Actinobacteria, Attini, FISH confocal microscopy, microbiota, Mollicutes, α -Proteobacteria

1 | INTRODUCTION

Several insect lineages have evolved farming symbioses with fungi. The oldest farmers are the platyponine ambrosia beetles (Smith, Kent, Boomsma, & Stow, 2018), which domesticated fungi ca.

90 MYA and later appear to have passed cultivars on to other clades of weevils (Vanderpool, Bracewell, & McCutcheon, 2018). The attine ants and macrotermite termites began to rear fungi considerably later, but have surpassed the ambrosia beetles by evolving large-scale farming comparable to culturally evolved human agriculture

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at an industrial scale (Aanen et al., 2002; Mueller & Gerardo, 2002; Nygaard et al., 2016; Schultz & Brady, 2008). Feeding up to millions within each colony, the ecological footprints of the fungus-growing ants and termites in, respectively, new and old world (sub)tropical ecosystems are substantial (Mueller & Gerardo, 2002; Wilson, 1971). Many details of these farming symbioses differ, however. Ants and termites are very different insects, they rear unrelated basidiomycete cultivar lineages, their founding colonies acquire crop symbionts in fundamentally different ways, and their fungus gardens are very differently structured (Mueller, Gerardo, Aanen, Six, & Schultz, 2005). Convergent evolution transformed feeding and foraging habits, and both systems independently evolved ways to upscale provisioning of fungus gardens with vegetable matter (Aanen & Boomsma, 2005; Schultz, Mueller, Currie, & Rehner, 2005). In doing so, the fungus-growing termites continued ancestral ingestion of dead plant material and ended up creating substrates for their fungus gardens from their primary faeces (Poulsen, 2015). However, the ancestors of the fungus-growing ants were at least partly predacious and thus had to convert to foraging for leaf-litter fragments and subsequently functional herbivory when the leaf-cutting ants arose (Branstetter et al., 2017; Schultz & Brady, 2008).

Recent research has increasingly focused on characterizing the symbiotic, usually gut-associated microbiomes of the fungus-growing ants and termites. The litter-feeding workers and minor soldiers of the Macrotermitinae have diverse bacterial gut microbiomes with decomposition functions complementary to those offered by the fungal cultivar, but royal pairs and soldiers have very low diversity gut microbiomes consistent with an exclusively fungal diet (Otani et al., 2014; Poulsen et al., 2014). Workers of the evolutionarily derived *Acromyrmex* leaf-cutting ants appear to have microbiomes in their guts and associated abdominal organs of comparable simplicity to those of fungus-growing termite royal pairs, consistent with all colony members having a specialized fungal diet (Sapountzis et al., 2015). These similarities suggest that food specialization reduces microbiome diversity, consistent with other ants and bees with specialized diets also having low diversity microbiomes (Anderson et al., 2012; Koch & Schmid-Hempel, 2011; Martinson et al., 2011). As such, endosymbiotic social insect microbiomes appear to be more distinct than those of vertebrates, where even specialized food is still related to very diverse microbiomes as, for example, in the Giant Panda (Xue et al., 2015), and in a study of fish which suggested a negative relationship between diet diversity and microbiome complexity (Bolnick et al., 2014). This overall impression is reinforced by two meta-studies in insects, which showed that hosts with more specialized diets tend to have less diverse gut microbiomes (Colman, Toolson, & Takacs-Vesbach, 2012; Yun et al., 2014).

The last two decades have seen a significant general research effort to elucidate the intricacies of the fungus-growing ant symbiosis. This work has documented that ant fungus farming evolved ca. 55–60 MYA and that the symbiosis underwent two subsequent transitions: one ca. 25–30 MYA, starting with the permanent domestication of a single fungal lineage that produced specialized hyphal tips (gongylidia) to feed the ants, and another ca. 15–20 MYA

when the leaf-cutting ants arose as active herbivores and evolved industrial-scale farming (Currie, Poulsen, Mendenhall, Boomsma, & Billen, 2006; Kooij, Aanen, Schjøtt, & Boomsma, 2015; Mueller et al., 2005; Mueller, Rehner, & Schultz, 1998; Nygaard et al., 2016; Schultz & Brady, 2008; Shik et al., 2014; Villesen, Murakami, Schultz, & Boomsma, 2002). It was also discovered that ant fungus farms have suffered from a single specialized genus of *Escovopsis* mycopathogens throughout their evolutionary history (Currie, 2001; Currie et al., 2006), which arose shortly after the attine ants started to maintain their crop fungi in dense garden aggregations (de Man et al., 2016; Gerardo, Jacobs, Currie, & Mueller, 2006; Yek, Poulsen, & Boomsma, 2012). This specific disease pressure is relevant because the attine ants evolved cuticular cultures of antibiotic-producing Actinobacteria to suppress *Escovopsis* and possibly other pathogens (Barke et al., 2010; Currie et al., 2006; Currie, Scott, Summerbell, & Malloch, 1999; Haeder, Wirth, Herz, & Spittler, 2009; Mattoso, Moreira, & Samuels, 2012; Seipke et al., 2011). Cuticular actinobacterial biofilms have been maintained by many, but not all attine genera, so that attine ants are exceptional in often having both an external (cuticular) microbiome and an internal abdominal microbiome (Sapountzis et al., 2015). Considering that the cuticular actinomycetes produce a range of antibiotics and antifungals (Barke et al., 2010; Holmes et al., 2016; Oh, Poulsen, Currie, & Clardy, 2009; Seipke et al., 2011; Seipke, Gruschow, Goss, & Hutchings, 2012) that are applied to fungus gardens that the ants ingest, it is conceivable that cuticular Actinobacteria may affect the composition and diversity of microbiomes in the guts and associated organs, but this hypothesis has remained untested.

Here, we present the first large-scale comparative analysis of abdominal microbiomes of attine fungus-growing ants, covering 16 Panamanian species, including data from a previous study focusing on three *Acromyrmex* species (Sapountzis et al., 2015). Our study encompasses eight of the 17 currently recognized attine ant genera (Sosa-Calvo et al., 2013; Sosa-Calvo, Schultz, Ješovnik, Dahan, & Rabeling, 2018) and spans the two stepwise domestication transitions in fungus farming, the first one from small-scale subsistence farming to specialized crop domestication and the final one that allowed industrial-scale herbivorous farming to emerge. We define abdominal microbiomes as the bacterial communities associated with the intestinal system consisting of both the digestive tract and the associated endocrine and excretory organs such as the fat bodies and Malpighian tubules. Strictly speaking, the abdomen of myrmicine ants (the subfamily to which the attine ants belong) contains only the terminal segments because the first abdominal segment is merged with the thorax and the second and third segments are constricted into the narrow petiole and postpetiole. We will nonetheless use the abdomen connotation here to mostly avoid the little-known ant-specific term “gaster.” We characterize the abdominal bacterial communities and examine their abundance, variability and stability across species, samples and abdominal organs to examine whether and to what extent: (a) Evolutionarily derived forms of fungus farming led to changes in the overall abundance of endosymbiotic bacteria and the degree of dominance of specific symbionts, and (b) the

composition and temporal stability of abdominal microbiomes is affected by the presence/absence of visible actinobacterial biofilms on the cuticle of worker ants.

A recent phylogenetic study (Branstetter et al., 2017) based on a powerful set of 1510 ultraconserved genomic elements has added considerable detail to our understanding of the biogeographic vicariance events that accompanied the transitions to more advanced forms of attine ant fungus farming. Ancestral state reconstructions showed that the evolution and domestication of gongyliidia-bearing cultivars happened in the dry habitats of South America away from the rainforests where fungus farming evolved, which helps to explain how the new cultivar lineage became reproductively isolated and started to co-evolve with the farming ants. The study also revealed that most of the evolutionarily derived generic lineages of the higher attines including the *Acromyrmex* and *Atta* leaf-cutting ants, evolved in Central/North America from a single colonizing lineage that reached the northern subcontinent ca. 20 MYA, that is, well before the Isthmus of Panama closed. Our present study was essentially completed before Branstetter et al. (2017) was published, but we explicitly analyse, interpret and discuss our results in the light of this new biogeographical frame of reference.

2 | METHODS

2.1 | Collections, dissections, DNA extractions and 16S-MiSeq sequencing

We used a total of 63 colonies from 14 fungus-growing ant species (this number increased to 76 colonies and 17 species after including data from 13 colonies of three *Acromyrmex* spp. previously published by Sapountzis et al. (2015), see also below). For the rare *Trachymyrmex* sp. 3 and *Cyphomyrmex rimosus*, we only found two and three colonies, respectively, and we only managed to obtain a single colony each of *Apterostigma cf. collare* and *Acromyrmex volcanus*. For all other ant species, we used four to six colonies, including two or three field colonies (for abdominal dissections or obtaining homogenates of entire abdomens) within a week of collection. These colonies were resampled after three months rearing in the laboratory, and similar samples were obtained from one to three colonies of the same species that had been collected on previous field trips and been maintained in the laboratory for more than two years (Supporting information Table S1). Ant species maintained in the laboratory were either fed with bramble leaves, dry rice and pieces of fruit (leaf-cutting ants), polenta (lower attines), or a combination of polenta and bramble leaves (*Trachymyrmex* and *Sericomyrmex*). To restrict variation in environmentally acquired bacteria, and to allow unbiased comparison between long-term laboratory colonies and a previous study (Sapountzis et al., 2015), we only used colonies collected in and around Gamboa, Republic of Panama, that were transferred to the same laboratory rearing rooms in Copenhagen and were maintained at ca. 25°C and 70% RH.

Our analysis focused on the bacterial communities associated with the digestive tract, the endocrine organs and the excretory

systems of the attine ants. To this end, we used dissected tissues (midguts, hindguts, Malpighian tubules and fat bodies) for most ant species and surface-cleaned entire abdomen (gaster) samples for species with workers that were too small to dissect. The latter samples also included cuticle material, some additional glandular tissue, some nervous tissue and the (normally inactive) ovaries, which only marginally affected our estimates of bacterial abundance (see below). Ant workers were anesthetized on ice, surface-cleaned by submerging them in absolute ethanol for 60 s and then rinsed with sterilized distilled water. We are aware that this external cleaning procedure will not have achieved 100% surface sterilization, but decided not to use more aggressive methods that could also degrade bacteria associated with internal tissues. This procedure also appeared reasonable in the light of a recent study (Bierer, Tysklind, Zinger, & Duplais, 2017) showing that workers of *Atta* leaf-cutting ants have very few cuticular bacteria, a result that we confirmed for several other attine species. In this context, it is important to note that cuticular biofilms of Actinobacteria on mature workers occur on the propleural (chest) plates and sometimes on surrounding thorax or leg body parts (Currie et al., 2006), so they will not have had direct effects on the abdominal microbiomes that we obtained. Dissections (only feasible for species with workers of at least ca. 5 mm body length) were done in sterile phosphate-buffered saline (PBS), pH 7.4 under a stereo microscope, after which tissues were stored at -80°C for later DNA extraction.

To make sure that our use of two sampling methods would not significantly affect our results, we also obtained a separate set of entire abdomen (gaster) samples from long-term laboratory colonies of the larger-bodied species. This allowed us to directly compare qPCR analyses of bacterial titres between entire ants and dissected abdominal samples for the species where dissections were feasible, that is, all species of *Acromyrmex*, *Atta*, *Sericomyrmex* and *Trachymyrmex* and *Apterostigma dentigerum*. To obtain these directly comparable samples, we collected abdomens from five workers from three colonies for the same species and partly the same colonies (Supporting information Table S1) and used the same qPCR methods to compare overall bacterial abundances (titres). For the remaining small-bodied species (*Cyphomyrmex* spp., *Mycocepurus smithii* and *Myrmicocrypta ednaella*), we used the already collected surface-cleaned worker abdomens as described above (Supporting information Table S1). We will henceforth use “dissected abdomens” when referring to dissected tissues and “entire abdomens” when referring to entire surface-cleaned body parts also known as ant gasters. This two-stage extra procedure allowed us to show that bacterial titres for entire abdomen samples were only slightly higher than for dissected samples so that biasing effects emerging from dissection constraints in small-bodied species have remained limited. We will further discuss these issues when presenting our results.

DNA was extracted from the frozen samples using the Qiagen Blood and Tissue kit following the manufacturer's instructions, and all samples were re-eluted in 150 µl AE buffer (Qiagen). We always included 1–2 blank “water” DNA extraction samples and quantified sample concentration using an ND-1000 spectrophotometer

(NanoDrop Technologies, Wilmington, DE, USA) before performing PCR reactions using 16S universal primers (Kozich, Westcott, Baxter, Highlander, & Schloss, 2013) to identify the optimal templates for library preparation and to confirm there was no detectable 16S amplification in the blank DNA extractions. Extracted DNA was sent to the Microbial Systems Laboratory at the University of Michigan for library preparation (multiplexed samples) and 16S-MiSeq paired-end sequencing, using the Kozich et al. (2013) protocol to amplify the V4 region including three blank “water” samples in the same MiSeq run.

The PCR mix contained 2.0 μ l 10 \times AccuPrime™ PCR Buffer II (15 mM MgCl₂), 0.15 μ l AccuPrime™ Taq DNA Polymerase (2 units/ μ l, Life Technologies), 1.0 μ l of each primer (10 μ M), 1.0 μ l diluted template and autoclaved MilliQ water (the same water used for the blank controls) to a total of 20 μ l. PCR incubation conditions included an initial activation of the hotstart polymerase at 95°C for 2 min, followed by 30 cycles of 95°C for 20 s, 55°C for 15 s, 72°C for 5 min, and final extension at 72°C for 5 min. For samples that did not amplify with this protocol (for a detailed list see Supporting information Table S1), we used an initial activation of the hotstart polymerase at 95°C for 2 min, followed by 20 cycles of 95°C for 20 s, 60°C for 15 s with a decrease of 0.3°C per cycle, and 72°C for 5 min, then 20 cycles of 95°C for 20 s, 55°C for 15 s and 72°C for 5 min, and a final extension at 72°C for 10 min (touchdown PCR). PCR products were purified using a magnetic bead capture kit (Ampure; Agencourt) and quantified using a fluorometric kit (QuantIT PicoGreen; Invitrogen). The purified amplicons were then pooled in equimolar concentrations and sequenced.

2.2 | Raw data analyses and OTU assignments

The MiSeq data were analysed using MOTHUR v1.33.3 (Schloss et al., 2009) after several rounds of filtering as described in the standard operating procedure (SOP; page accessed July 2014) with a few modifications (Kozich et al., 2013). We also included data that were part of a previous publication (Sapountzis et al., 2015), which had been sequenced in the same MiSeq run. Contigs were created using the *make.contigs* command and were subsequently trimmed to remove sequences exceeding 275 bp or having ambiguous base pairs. We then identified unique sequences and aligned them against a customized database originating from a SILVA nonredundant database (version 111, accessed July 2014), which had only the V4 region of the 16S rRNA gene. Sequences that did not cover positions 13,862–23,444 of the full-length SILVA alignment (V4 hypervariable region) or had a homopolymer length above 8 were removed. Overhangs on the sequences were also removed using the *filter.seqs* command and all sequences were screened again in order to retain only the unique ones. Chimeric OTUs were removed before sequences were assigned to taxonomic groups using the Bayesian classifier implemented in MOTHUR with a confidence threshold of 80% against the same SILVA database used for the alignment, after which any reads belonging to Archaea, Eukaryota, chloroplasts or mitochondria were removed.

OTUs were identified at 97% similarity and all singletons were retained for further analysis, since there is no objective way to distinguish between rare but important OTUs representing true diversity and contaminants or sequencing errors. Rarefaction curves were visualized (Supporting information Figure S1) using the R package INEXT (Hsieh, Ma, & Chao, 2016), and rarefaction tables were constructed with MOTHUR using pseudoreplicate OTU data sets containing between 1 and 122,998 sequences, with 1,000 iterations per pseudoreplicate. The final OTU table was rarefied at 8,900 reads, which reduced the number of OTUs from 3,334 to 2,303 and was used for any downstream analyses that did not exclusively consider the most abundant OTUs having prevalences (relative abundance) of >10% in at least two samples across the entire data set (Supporting information Figure S2). Following Hu et al. (2017), we identified the OTUs present in the blank “water” samples that were sequenced along with the real samples, and removed OTUs as contaminants if their relative abundance produced an average blank/sample ratio ≥ 0.2 . We also performed an extra set of analyses focusing on the unique sequences in our data set, using a variety of filtering procedures to validate that the relatively high bacterial diversity that we obtained for many of the lower attine abdominal samples was not an artefact associated with having low amounts of bacterial DNA (available at <https://doi.org/10.5061/dryad.tj30d>), a known possible complication of MiSeq sequencing studies (Salter et al., 2014). This further reduced the number of OTUs to 3,103 (unrarefied) and 2,099 (rarefied).

For the maximum-likelihood phylogenetic tree, the 16S sequences were extracted from the MiSeq data using the *get.oturep* command in MOTHUR. Sequences were aligned using MUSCLE v3.8.31, the alignments were checked with JMODELTEST v2.1.7, and the phylogenies were built with RAXML v8.2.11 using the “rapid bootstrap and best-scoring ML” algorithm with 1,000 bootstraps after further modification in FIGTREE v1.4.2.

2.3 | Comparisons across phylogenetic farming transitions, sampling categories and presence/absence of cuticular Actinobacteria

The richness, diversity and composition of the specific abdominal bacterial communities were compared based on phylogenetic position of the ants and two additional partitions. Phylogenetic signals in the data set were examined based on a modified version of the tree presented by Schultz and Brady (2008), incorporating also data from Mehdiabadi, Mueller, Brady, Himler, and Schultz (2012), Ješovnik, González, and Schultz (2016) and Nygaard et al. (2016), and assuming that *S. amabilis* is a close relative of *Sericomyrmex cf. parvulus*. Branch lengths were based on the divergence times estimated by Schultz and Brady (2008), and between-colony variation within each species was entered as a polytomy at 1 MYA. This within-species divergence time represents approximately half the observed divergence time of the most closely related sister species in the tree and was considered to be a reasonable compromise between likely overestimating the divergence time between colonies

and likely underestimating random variation between colonies not due to common ancestry. The tree was then used to obtain phylogenetic signals in overall bacterial abundances (qPCR data, see below) and alpha diversity (species richness, diversity and inequality) and to produce a matrix of pairwise divergence times between ant species to examine beta diversity.

Additionally, we partitioned samples based on the environmental conditions that colonies experienced at the time of sampling, that is, whether workers were collected in the field, from colonies ca. 3 months after establishment in the laboratory (short-term laboratory) or from colonies that had been kept in the laboratory for >2 years (long-term laboratory). We refer to this partitioning as sampling environment. The final partitioning that we used was between those species whose workers have clearly visible cuticular Actinobacteria on at least some body parts, and those that do not, since these Actinobacteria produce antibiotics that can potentially influence the composition and diversity of endosymbiotic microbiomes. The effects of these two partitions on measures of alpha and beta diversities were always examined while statistically removing any phylogenetic signal present.

2.4 | Diversity analyses

Many different measures of alpha diversity for samples of communities have been proposed. The simplest of these is richness—the number of species (or in our case, OTUs) observed in a sample, S_{obs} —but this does not take into account differences in prevalence of OTUs within samples. Richness can be decomposed into independent “true diversity” and “inequality” components (Jost, 2010), with a common family of measures of “true” diversity putting increasing emphasis on common rather than rare OTUs based on what is referred to as Hill numbers (Hill, 1973). A Hill number of 0 gives S_{obs} , a Hill number of 1 is the exponential of Shannon entropy (e^H), and a Hill number of 2 is the inverse Simpson diversity index, etc. We therefore calculated both S_{obs} and e^H for each sample as measures of diversity in MOTHUR (Schloss et al., 2009), together with their related measures of inequality (S_{obs}/e^H ; equivalent to the exponential of Theil entropy (Theil, 1967), e^T), and compared these across samples. To avoid confusion between the two entropy terms, we will refer to Shannon entropy as Shannon diversity and to Theil entropy as Theil inequality in the remainder of this study. In addition, we also examined phylogenetic diversity (Faith, 1992) of bacterial OTUs across samples, to weight OTU richness by phylogenetic distance between bacterial OTUs. For analyses, each index was log-transformed to normalize its distribution and reduce heteroscedasticity (i.e., the indices used were $\log_e S_{\text{obs}}$, H , T and $\log_e PD$). The *phylosig* command within the R package PHYTOOLS (Revell, 2012) was used to test whether there was any significant phylogenetic signal in these indices. This procedure computes a measure of phylogenetic signal, K (Blomberg, Garland, & Ives, 2003), and tests the probability that it could arise by chance with the selected data set using 100,000 randomizations across the phylogeny. Changes in the rate of evolutionary change in each index across the phylogeny were modelled using a Bayesian Markov chain

Monte Carlo (MCMC) approach, using a Brownian model of character evolution, as implemented in the *evol.rate.mcmc* command in PHYTOOLS, which identifies the phylogenetic location of shifts (splits) in the evolutionary rate through time (Revell, Mahler, Peres-Neto, & Redelings, 2012). Default values were used for this command, including uniform priors, but with the number of MCMC generations increased to 100,000 to ensure model convergence. The split representing the position of the largest transition in evolutionary rate in the tree was then identified using the *MinSplit* command on the model output. Using the same phylogeny and diversity indices, we also identified the most pronounced additional splits using the SURFACE package in R, which identifies all significant transitions across the phylogeny based on an Ornstein–Uhlenbeck model of character evolution and stepwise Akaike information criteria (Ingram & Mahler, 2013). The explanatory power of the intermediate split that we obtained empirically (referred to as “A-transition”), relative to the “traditional” transitions from lower-to-higher and higher-to-leaf-cutting attines, was evaluated by comparing the Akaike weights of linear mixed models in which the maximally supported split and the two traditional splits were included as explanatory variables, with ant species nested as a random factor within the resultant groups. We then used the evidence ratios of the Akaike weights of each combination of two models as estimates of how many times better the maximally supported split performed relative to the alternative traditional splits.

Differences in bacterial richness, diversity and inequality between sampling environments and the presence/absence of cuticular Actinobacteria were examined using a Bayesian (MCMC based) generalized linear mixed model as implemented in the R package MCMCGLMM (Hadfield, 2010). This approach allows a phylogenetic tree to be included in the model as a random effect, so that the fixed effects can be examined independent of any phylogenetic signal (Hadfield & Nakagawa, 2010). For each index, five models were constructed, including all possible combinations of sampling environment (E) and the presence or absence of Actinobacteria (A) as main effects, and their possible interactions (i.e., E + A + E × A; E + A; E; A; and only random effects), after which the best model based on the AICc values was selected with the R package MUMIN (Bartón, 2017). MCMCGLMM estimates a significance level for the comparison between a reference category and each other category within categorical main effects, so for sampling environment the short-term laboratory category was chosen as the reference level, as it allowed direct comparison of both changes associated with transfer from the field to the laboratory and changes associated with long-term laboratory culture.

To examine the differential representation of particular OTUs between different types of partitioning of samples, we used the DESEQ2 program in R (Love, Huber, & Anders, 2014). We also examined similarities and differences in OTU community (beta diversity) between pairs of samples to validate whether: (a) Any significant transition in overall bacterial abundance that we uncovered did in fact concern largely the same OTU composition or involved a significant discontinuous shift in OTU identities, and (b) whether transfer of colonies to the laboratory might have caused general shifts of this

kind in overall OTU compositions because there might exist typical laboratory-invasive strains that could become more generally abundant while they were absent or rare in the field.

We used three different measures to quantify pairwise bacterial community dissimilarity between samples: Bray–Curtis dissimilarity and weighted and unweighted UniFrac distances (Lozupone & Knight, 2005). To visualize differences in beta diversity (changes in community composition), and to reduce the number of variables for subsequent discriminant analyses, the matrices of pairwise dissimilarities between samples were ordinated using two-dimensional nonmetric multidimensional scaling (NMDS) with the *metaMDS* command, and plotted using the *ordiplot* command in the R package *vegan*. The NMDS stress, which shows how well the samples are represented by two axes, was calculated using the “stressplot” command and is given in each ordination plot. All but one of these stress values were ≤ 0.2 suggesting good representation of the variation by 2 axes and the remaining one was only slightly higher. We carried out linear discriminant analyses based on the NMDS scores to evaluate how well ant species could be separated based on their bacterial OTU communities retrieved from sampling replicate colonies, within the two partitions of phylogeny and presence/absence of cuticular Actinobacteria that we used. This produced estimates of the percentage of samples assigned to the correct ant species, which were further evaluated by the associated Entropy R^2 value, a measure of discriminatory power with higher values indicating better discrimination. To examine differences in the average similarity of abdominal bacterial community samples across partitions (basal vs. evolutionarily derived attines; cuticular Actinobacteria present or absent; field vs. short-term laboratory vs. long-term laboratory collections), we used PERMANOVA (permutational multivariate analysis of variance) based on the pairwise dissimilarity matrices with the *adonis* command in *vegan*, using 9,999 permutations. Finally, we explicitly assessed differences in the variability in bacterial communities between ants with or without cuticular Actinobacteria and between the best-supported phylogenetic split (“A-transition”) identified in our analysis, because these differences remain implicit in discriminant analysis and are potentially relevant if phylogenetic transitions or shifts in the presence/absence of cuticular Actinobacteria would imply that abdominal bacterial communities become specialized on not only different but also fewer OTUs. For this analysis, we used the HOMOVA command in *MOTHUR* based on the pairwise dissimilarity matrices, comparing homogeneity of variance between pairs of partitions of the complete set of samples, and applying Bonferroni-adjusted alpha-values because of multiple tests across the same data matrix.

2.5 | Fluorescent in situ hybridization (FISH) microscopy

To start understanding what putative functions the most abundant bacterial endosymbionts may have, it is important to visualize them in the organs they are primarily associated with. We therefore used probes from a previous study (Sapountzis et al., 2015) and designed several more (Supporting information Table S2) so we could target most of the abundant OTUs (according to our double $>10\%$ presence

criterion) and use FISH microscopy to identify their main locations along the intestinal system and its associated organs. Four of these abundant OTUs (*Rhizo19*, *Solir27*, *Rhizo30* and *Rhizo35*) were only detected in field samples and thus had to be excluded for FISH microscopy. We chose this specific FISH approach instead of using 16S EUB probes that target all bacteria, because it allowed us to use: (a) two specific types of probe (OTU- or class-specific) and (b) DAPI staining as a positive control to detect all bacteria in the same sample. The procedure for designing probes was that OTU sequences were retrieved using the *get.oturep* command implemented in *MOTHUR* and aligned using the Geneious Custom Assembly algorithm, after which probes could be designed using *GENEIOUS R7 v7.0.6* (Kearse et al., 2012). OTU representative sequences were then compared with each other and with the newly designed probes using the *BLAST* algorithm (Altschul, Gish, Miller, Myers, & Lipman, 1990) against the nonredundant *SILVA* database using the web interface, and against a local database that was created using the *makeblastdb* command with a *FASTA* file containing all the representative OTU sequences identified in this study. Probes were further tested with an online tool (OligoAnalyzer 3.1, Integrated Data Technologies) to check for hairpins and self-dimers (Supporting information Table S2).

Six-to-ten ant workers from several short-term and long-term laboratory colonies belonging to 10 of the 17 attine species used for 16S-MiSeq analysis (*At. colombica*, *At. cephalotes*, *T. cornetzi*, *T. zeteki*, *S. amabilis*, *C. costatus*, *C. longiscapus*, *Myc. smithii*, *Myr. ednaella* and *Ap. dentigerum*) were dissected in PBS and their gut, endocrine and excretory tissues (midgut, fat bodies, Malpighian tubules, ileum and rectum) were placed in 4% paraformaldehyde for at least 24 hr (see Supporting information Table S1 for details of colonies used). The localizations of the OTUs of interest in *Ac. echinator* and *Ac. octospinosus* have been characterized in a previous study (Sapountzis et al., 2015) and are therefore not presented here. Half of the worker tissue samples were used for hybridization with OTU-specific or class-specific probes, and the other half for hybridization with antisense probes (reversed sequences) serving as negative controls. A single microscopy sample always consisted of tissues from three to five ant workers from the same colony. For the permeabilization, deproteinization and hybridization (using $0.75 \mu\text{g}/\mu\text{l}$ of our 16S-specific labelled probes; Supporting information Table S2), we followed a previously established protocol (Sapountzis et al., 2015). All slides were observed and photographed using a Zeiss LSM 710 laser-scanning confocal microscope equipped with ZEN 2009 software and a Leica TCS SP2 microscope. Images were processed with Adobe Photoshop CS5 for publication to merge the three channels and add scale bars. Merged RGB images were subsequently processed with ImageJ to evaluate the morphology of the suspected positive bacterial signals (granulometric filtering) and with a customized JavaScript to create frames showing special regions of interest (ROIs).

Scoring microscope image signals against background autofluorescence is a semiquantitative manual procedure that precludes formal statistical comparisons. Following Sapountzis et al. (2015), we used very strict criteria for accepting positive signals as genuine. We only considered signals that colocalized with DAPI signals

of bacterial size (ca. 2 μm) and which were visible in only one of the probe channels. This is a reliable signal quality check because we never stained the same bacterial OTUs with different fluorochromes, so signals appearing in both probe channels would imply either autofluorescence or unspecific staining. We confirmed that our experimental protocol did not allow for unspecific binding of probes by using same-tissue images stained with matching antisense probes, which showed no specific staining of the 16S antiprobes but only DAPI signals. The same process was used in an earlier study, which confirmed high probe specificities with electron microscopy (Sapountzis et al., 2015).

2.6 | Validating FISH observations on overall bacterial abundances with quantitative PCR

To quantify the relative bacterial 16S abundance levels for each of the ant species, qPCR was carried out on samples of pooled entire abdomens (gasters) from each of the species used for FISH (Supporting information Table S1). The primers used for the MiSeq library construction (515F/806R; obtained from Kozich et al. (2013)) were also used for the qPCR reactions, which were performed as described by Sapountzis et al. (2015). All qPCR reactions were replicated twice, and the Ct (cycle threshold) mean across replications was used as a measure of amplicon abundance relative to two negative controls of no added template. Cell count data were normalized relative to the ant nuclear gene *elongation factor 1 alpha* (*EF-1 α*) (Andersen, Boye, Nash, & Boomsma, 2012), and standard curves with PCR products in tenfold dilution series of known concentration were used for both 16S and *EF-1 α* . The Ct values of the standards were then used to calculate PCR efficiency using the software REST v3.04 (Pfaffl, Horgan, & Dempfle, 2002), after which data were expressed as fold changes using a previously described formula for relative quantification (Pfaffl, 2001), with the mean of the three *Ap. dentigerum* samples (the most basal branch in the phylogeny) as reference (Adent_f1, Adent_3m1 and Adent_3m2: supporting information Table S1). The phylogenetic signal in overall bacterial abundances and how phylogenetically independent abundances differed between laboratory and field-collected samples, and between species with and without cuticular Actinobacteria, were analysed using the same procedures as for alpha diversity.

3 | RESULTS

3.1 | Identification of abdominal bacteria across the phylogeny of fungus-growing ants

Our analyses of 107 samples from 76 Panamanian colonies (including 19 *Acromyrmex* samples from 13 colonies from Sapountzis et al., 2015) retrieved 3,334 bacterial OTUs (operational taxonomic units = Putative species), including singletons, at the 97% sequence identity cut-off, across 48 bacterial classes. After rarefaction and filtering of “water” OTUs (removed when present in the negative controls; see Methods), this number was reduced to 2099 (Supporting

information Figure S1 and Table S3). The most prevalent of these were Mollicutes (334,358 hits; >35% of all reads; 16 OTUs), α -Proteobacteria (306,740 hits; >32%; 252 OTUs), Actinobacteria (134,031 hits, >14%, 240 OTUs) and γ -Proteobacteria (80,281 hits, >8%; 143 OTUs) (Figure 1 and Supporting information Table S3). Rarefaction curves showed that sampling had been adequate except for a single field sample of *C. rimosus* (Supporting information Figure S1), which we nonetheless included in the analyses as the effect of undersampling was generally conservative (i.e., differences would have been higher if more OTUs had been sampled). To facilitate reading and interpretation, we have named the 98 most abundant OTUs (OTUs0001–0100 based on Mothur's relative abundance ranking, using five letters that refer to their bacterial order followed by a specific number that identifies their overall rank (Supporting information Figures S2 and S3; Table S3). This produced a list of 98 OTUs rather than 100, because rarefaction at 8,900 reads eliminated two OTUs.

Before using these comparative data to evaluate diversity patterns, we examined potential confounding effects in our sample preparation methods that could have emanated from: (a) the initial analyses having used both standard PCR (84 samples) and touchdown PCR (23 samples) to generate the libraries, (b) pools of five ant workers (most species) and pools of 10–15 workers (some very small lower attine ant species) having to be compared, (c) the presence of potential laboratory-borne contaminants and (d) the unavoidable lower biomass of some samples (Salter et al., 2014). Ordination and matching statistical tests showed that we could not reliably exclude that the number of individuals sampled and particularly the PCR method used might have affected our bacterial richness scores and alpha diversity estimates (Supporting information Results S1). These complications, together with the unknown likelihood that some contaminations might not have been filtered out because we did not run a high number of blank samples, imply that we present our alpha diversity estimates with some precaution, and that we based our remaining analyses on the 84 samples that were amplified with standard PCR. This meant that a single species (*Apt. collare*) for which we only had a single sample that had been amplified with touchdown PCR has not been considered further, so the number of species dropped from 17 to 16 in most analyses.

To ensure that our emphasis would focus on bacteria with the highest likelihood of having functional significance for ant metabolism and performance of the fungus farming symbioses as a whole, we identified the limited set of OTUs with prevalences of more than 10% in at least two samples—a criterion that emphasizes relative abundance while avoiding that single occurrences are decisive. Our initial analyses based on 107 samples identified 21 OTUs that met this criterion, which accounted for more than 83% of the total reads, a number that was reduced to 18 OTUs accounting for more than 90% of the total reads when we only considered the 84 samples amplified with standard PCR (Supporting information Figure S3 and Table S3) because touchdown PCR tends to generate more spurious sequences. Six of these 18 or 21 abundant OTUs have previously been identified in the guts and associated organs of three species

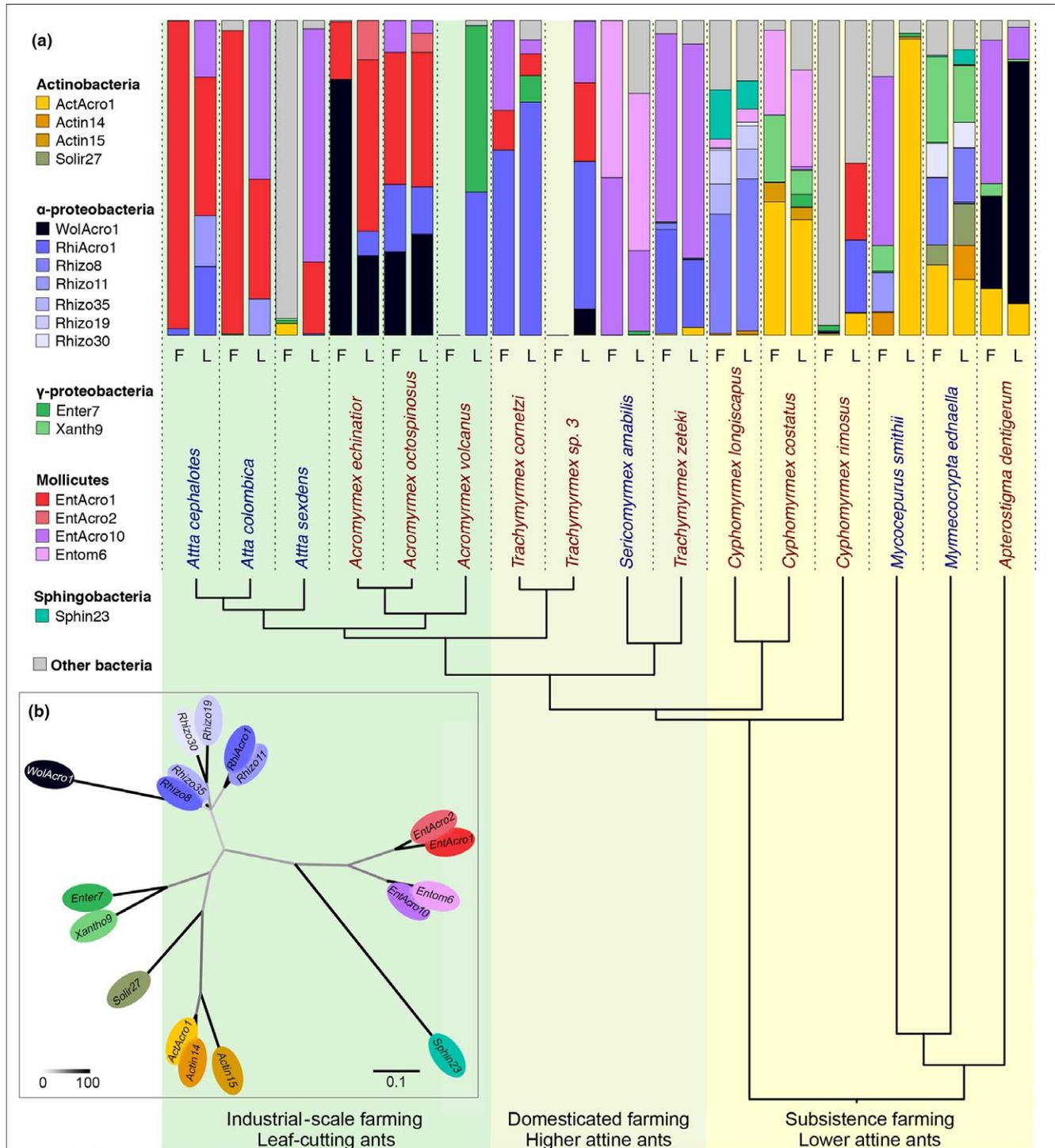


FIGURE 1 Relative abundances (prevalences) of the 18 abundant abdominal bacterial OTUs across 16 Panamanian fungus-growing ants based on 84 samples for which libraries were generated after amplification with standard PCR. (a) The host phylogenetic tree has been modified from (Branstetter et al., 2017; Schultz & Brady, 2008)—see text and Supporting information Figure S4. Known farming transitions are highlighted by background colour shifts from yellow to light green to darker green. Vertical bars only include the 18 OTUs that reached $\geq 10\%$ in at least two ant samples (see colour legend) and a single pooled prevalence of all other OTUs (grey). The left bar for each species refers to field-collected samples (F) and the right bar to ants sampled after colonies were maintained in the laboratory (L), pooling short-term and long-term laboratory colonies. Relative abundances were calculated using all 2099 OTUs after rarefaction and averaged across all field or laboratory samples for each species. Species names are coloured according to whether worker ants have visible cuticular Actinobacteria (red) or not (blue). The phylogenetic relationships of the 18 abundant OTUs can be seen in Supporting information Figure S7. (b) A maximum-likelihood nonrooted phylogeny showing the relationships of the 18 abundant OTUs identified in our study (following the $>10\%$ prevalence criterion in at least two of the 84 samples amplified with standard PCR) with ellipses in the same colours as the bars in the A-panel. The scale bar at the bottom illustrates how branch lengths reflect substitution rates, and the heatmap bar represents degree of bootstrap support

of *Acromyrmex* leaf-cutting ants from the same sampling site, and retained their names from that study (*ActAcro1*, *EntAcro1*, *EntAcro2*, *EntAcro10*, *RhiAcro1* and *WolAcro1*: Sapountzis et al., 2015).

Distribution patterns of OTUs across the phylogenetic tree are given in Figure 1 and Supporting information Figure S3, arranged according to the phylogenetic relationships between the 16 attine species. The four dominant bacterial classes were all represented in the lower attine ants, but the higher attine ants had generally endosymbiotic microbiomes dominated by fewer OTUs that belong more predominantly to the Entomoplasmatales (Mollicutes) and Rhizobiales (α -Proteobacteria). In contrast, Actinobacteria were prevalent abdominal endosymbionts in many lower attines, but were essentially lacking in the higher attines and leaf-cutting ants. The basal higher attine ant species *Trachymyrmex* (but not the *Sericomyrmex*) have a Rhizobiales symbiont (*RhiAcro1*) not found in the lower attines (with the exception of *C. rimosus*). Shifts in endosymbiotic abdominal OTUs continued in the leaf-cutting ants, with the genus *Acromyrmex* having Entomoplasmataceae (*EntAcro1* & *EntAcro2*) rather than the typical Spiroplasmataceae (*Entom6* & *EntAcro10*) of *Trachymyrmex* and *Sericomyrmex*, and abundant *Wolbachia* (*WolAcro1*; α -Proteobacteria) otherwise only found in the most basal attine *Apt. dentigerum*. Enterobacteriaceae (*Enter7*; class: γ -Proteobacteria) also appear here in a single species (*Ac. volcanus*; the same OTU as in *T. cornetzi* and *C. costatus*). Finally, across the *Atta* species, we found the same Spiroplasmataceae (*EntAcro1* & *EntAcro2*; class Mollicutes) as in *Acromyrmex* but a different common Rhizobiales OTU (*Rhizo11*) because *RhiAcro1* was largely absent, with the exception of most colonies of *Ac. cephalotes*. *Wolbachia* was only found in traces across *Atta* species, except for a single *Atta sexdens* field colony where *Wolbachia* reached higher prevalence.

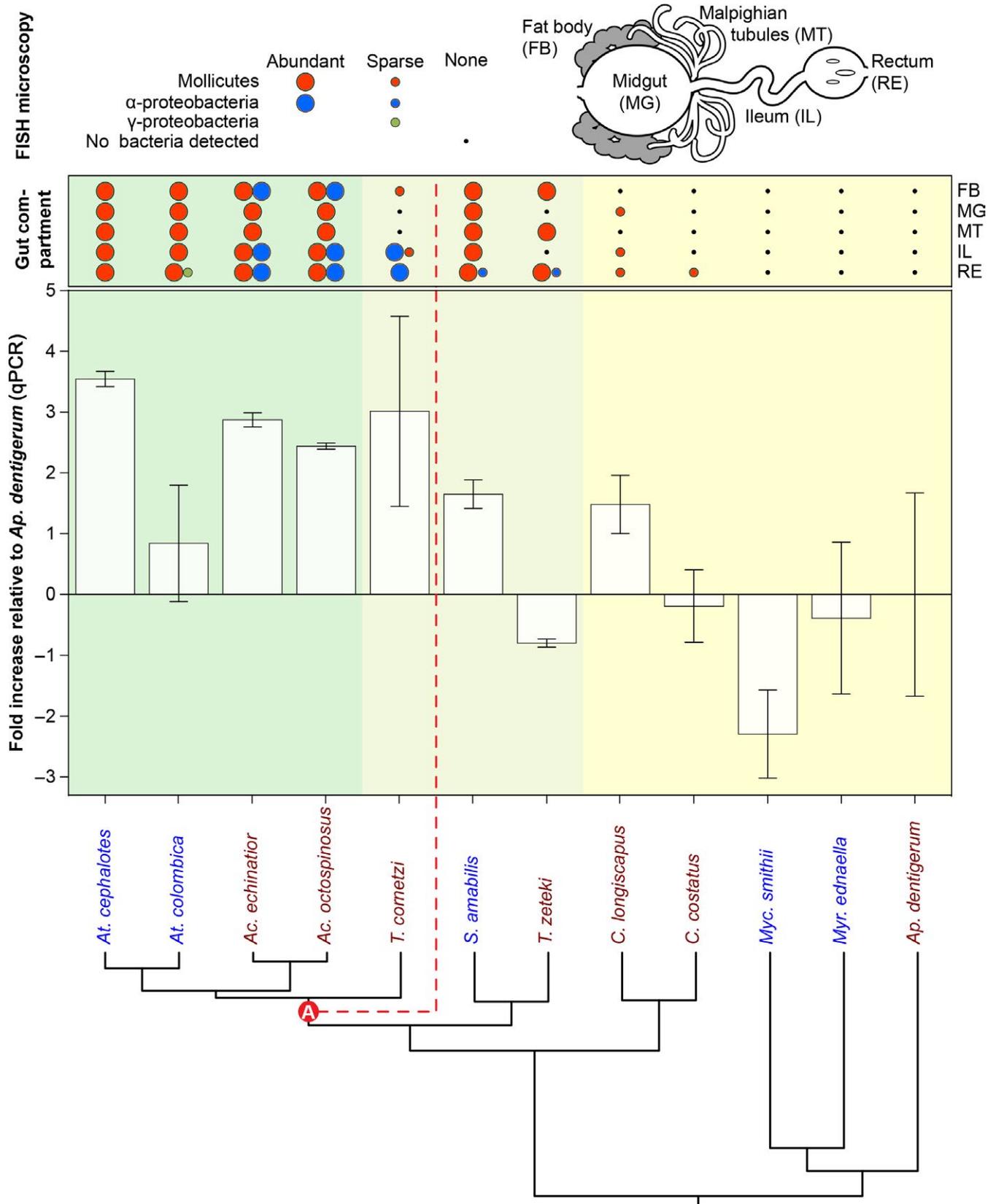
Our analyses of alpha diversity across the 84 samples indicated that OTU richness ($\log_e S_{\text{obs}}$), diversity (Shannon diversity H), inequality (Theil inequality T) and phylogenetic diversity ($\log_e PD$) of abdominal bacteria all showed a significant phylogenetic signal ($\log_e S_{\text{obs}}$: $K = 0.0847$, $p < 0.0001$; H : $K = 0.0515$, $p = 0.0022$; T : $K = 0.0588$, $p < 0.0001$; $\log_e PD$: $K = 0.0829$, $p < 0.0001$) (Supporting information Figure S4 and Table S4). However, the branch in the phylogenetic tree at which the rate of change was highest differed between the different indices and types of analyses: The *minsplit* analysis identified the largest split within *S. amabilis* (Shannon diversity H), between the paleo-attines (*Myrmicocrypta*, *Mycocyperus*, *Apterostigma*) and the remaining (*Cyphomyrmex*, *Trachymyrmex*, *Sericomyrmex*) lower attines (Theil inequality T) and between *C. rimosus* and the other *Cyphomyrmex* spp. ($\log_e S_{\text{obs}}$, $\log_e PD$), while “SURFACE” analysis suggested an important transition within *S. amabilis* (Shannon diversity H), between higher and lower attines ($\log_e S_{\text{obs}}$) and within the *Trachymyrmex* grade (Theil inequality T , $\log_e PD$). We will refer to the latter as transition “A” (see Supporting information Figure S4 and qPCR results given below and in Figure 2). Although there is substantial variation across the phylogenetic branches, the overall statistics indicate that there is a trend towards lower alpha diversity when going from the base to the crown of the attine ant tree. However, we do not consider this to be a hard result because our validations indicated that our alpha

diversity estimates were likely influenced by a number of confounding effects (Supporting information Results S1).

3.2 | OTU localization by FISH and estimates of overall bacterial abundances using qPCR

FISH microscopy showed that abundant Mollicutes OTUs were widespread across many abdominal tissues in both *Acromyrmex* and *Atta* leaf-cutting ants (Supporting information Results S2; Sapountzis et al., 2015), but that the abundant Rhizobiales OTU (*RhiAcro1*) in *Acromyrmex*, often co-occurring with *Wolbachia* (Andersen et al., 2012; Sapountzis et al., 2015), was never detected with specific probes in worker tissues of the *Atta* colonies that we examined (Figure 2), consistent with the MiSeq data plotted in Figure 1 and Supporting information Figure S3 showing that *RhiAcro1* is rare in *Atta* colonies. However, the *RhiAcro1* OTU was as highly abundant in the hindgut of *T. cornetzi* as in *Acromyrmex* species (Sapountzis et al., 2015), but Mollicutes signals in *T. cornetzi* were sparse and restricted to the fat body and ileum (Supporting information Results S2; Figure 2). In both *S. amabilis* and *T. zeteki*, which belong to a phylogenetically more basal lineage of higher attine ants (Branstetter et al., 2017; Schultz & Brady, 2008; Sosa-Calvo et al., 2018), there were very few *RhiAcro1* signals, but Mollicutes OTUs could be stained in most of the tissues examined (Figure 2). It is important to note, however, that these Mollicutes were a different OTU (*EntAcro10*) than the *EntAcro1* bacteria that were exclusively found in *Atta*, *Acromyrmex* and *T. cornetzi* (Figure 1; Supporting information Figure S3 and Table S3). We detected no Actinobacteria signals in the abdominal tissues of *S. amabilis* and *T. zeteki* even though the MiSeq sequencing showed a low relative abundance of *ActAcro1* across the 15 colonies of *S. amabilis* and *T. zeteki* examined (usually <3%–4% but sometimes as high as 14% in a single laboratory colony; Tz_11; Table S3; using all 107 samples as this result is independent of confounding effects). We never detected any endosymbiotic Actinobacteria in the abdomens of the evolutionarily more derived *T. cornetzi*, *Acromyrmex* and *Atta* with FISH, consistent with them being absent or sparse (Table S3) in spite of *T. cornetzi* and all three *Acromyrmex* species maintaining cuticular biofilms in which *ActAcro1* is the dominant OTU (Andersen, Hansen, Sapountzis, Sørensen, & Boomsma, 2013).

Our FISH survey only rarely detected general bacterial signals in abdominal tissues of *Cyphomyrmex* and *Myc. smithii* workers and we never observed specific signals in abdominal tissues of *Myr. ednaella* or *Ap. dentigerum* (Figure 2, Supporting information Results S2). These absences were confirmed using a wide range of specific FISH probes, and neither could bacterial DNA be stained with DAPI in replicated samples of multiple colonies of these species (Figure 2, Supporting information Results S2). The qPCR analyses of pooled entire abdomens (gasters) across the attine ants for which we obtained FISH data (Table S5) confirmed that overall bacterial titres in lower attines were low and part of a strong phylogenetic signal of increasing bacterial titres towards the crown of the phylogenetic tree ($K = 0.113$, $p = 0.001$; Figure 2). This change in overall bacterial abundances was not a smooth gradient, but had an unusually steep increase at the “A” transition within the



Trachymyrmex grade that we defined above based on two alpha diversity measures also highlighting this transition (see previous section and Supporting information Figure S4). The fold-increase in bacterial 16S DNA relative to that found in *Ap. dentigerum* (standardized to the ant

housekeeping gene EF-1 α) was 2.54 ± 0.56 above the "A"-transition and -0.08 ± 0.48 below, indicating that bacterial titres essentially remain unchanged until they realize a stepwise increase to higher abundance at the "A" transition. This transition explained differences in bacterial titre

FIGURE 2 Summary of gut partitions where bacterial OTUs could be localized with fluorescence in situ hybridization (FISH) microscopy and overall bacterial abundances measured as 16S rRNA copies in homogenized abdomens of 12 attine ants using qPCR, ordered from crown to base of the attine phylogenetic tree. QPCR data are presented as the ratio of the total number of bacterial 16S rRNA gene copies relative to the number of ant *EF-1a* gene copies per sample and plotted relative to the mean value for the phylogenetically basal *Ap. dentigerum* samples as calculated by the method of Pfaffl (2001). Results are shown as means \pm SE, based on three colony-level DNA samples for each ant species. The phylogeny is a 12-branch extract of the tree shown in Figure 1. The steepest observed transition in overall (total 16S copies) abundance is marked as transition “A.” A similar analysis using qPCR data without normalization comparing entire and dissected abdominal samples produced essentially the same results (supporting information Results S3). Above each histogram bar, the presences or absences of 13 of the 18 abundant OTUs for which FISH probes were available are plotted in two qualitative abundance categories reflecting whether FISH signals were consistently detected or rarely present. The 16S-FISH confocal microscopy was done separately for fat body cells, midgut, Malpighian tubules, ileum and rectum (see schematic diagram at the top right and Supporting information Results S2 for details)

much better than the traditional transitions from lower attine subsistence farming to higher attine domesticated farming and to industrial-scale herbivorous leaf-cutting farming (evidence ratio = 6.00).

The apparent “A” transition in overall abundance of abdominal endosymbiotic bacteria prompted us to further evaluate the extent to which the composition and distribution of bacterial communities might also reflect this transition. Using our original data set of 3,103 unrarefied OTUs belonging to 48 bacterial classes and the DESEQ software for analysis (Love et al., 2014), it appeared that, at the level of bacterial classes, Actinobacteria, Betaproteobacteria, Clostridia and Sphingobacteria were significantly more prevalent in the abdomens of the attine species basal to the “A” transition, while Alphaproteobacteria were more prevalent beyond the “A” transition (DESEQ adjusted $p < 0.001$ in all contrasts). At the OTU level, *EntAcro1* (Mollicutes) and the nonabundant *Xanth21* (Gammaproteobacteria) and *Rhodo286* (Alphaproteobacteria) were significantly more prevalent in the evolutionarily derived attine lineages (DESEQ adjusted $p < 0.001$, $p < 0.001$ and $p = 0.03$, respectively; Figure 3 and Supporting information Table S6), while 19 other OTUs belonging to diverse bacterial classes were significantly more prevalent in the basal attine lineages (Figure 3 and Supporting information Table S6). Of the 22 OTUs that showed significant differences in proportional representation (Supporting information Table S6), nine were among the 18 most abundant OTUs previously identified with our double $>10\%$ criterion. Remarkably, *ActAcro1*, the dominant Actinobacteria on the cuticle of *Acromyrmex* species in Gamboa (Andersen et al., 2013), was one of the significantly underrepresented OTUs in the abdominal tissues of this evolutionarily derived group of higher attine ants, indicating that this OTU is only a relatively common endosymbiont associated with guts and abdominal organs throughout the basal attine lineages (lower attines plus *T. zeteki* and *S. amabilis*) where the overall bacterial abundances tend to be low (Figure 2 and Supporting information Table S6).

3.3 | Sensitivity of abdominal microbiomes to cuticular Actinobacteria and sampling environment

After phylogenetic effects had been removed, there was no overall association between the presence/absence of Actinobacteria and bacterial OTU richness (MCMGLMM: $p = 0.873$; Supporting information Table S7A), but colonies that had been kept in the laboratory for 3 months generally had abdominal microbiomes with lower richness than field colonies ($p = 0.007$), although they did not differ in richness from those kept in the laboratory for several years ($p = 0.222$;

Supporting information Table S7A). PD (Faith's Phylogenetic Diversity) followed the same pattern (Actinobacteria presence: $p = 0.274$; short-term laboratory vs. field: $p = 0.0109$; short-term laboratory vs. long-term laboratory: $p = 0.66$; Supporting information Table S7B). For the other alpha diversity indices, there were no significant associations with either the presence/absence of cuticular Actinobacteria or sampling environment (Supporting information Table S7C,D).

We subsequently examined changes in specific OTUs between field and laboratory colonies, to see whether transfers systematically favoured the prevalence of some OTUs over others. This showed that *Atta* leaf-cutting ant species tended to gain the dominant OTUs *EntAcro10*, *RhiAcro1* and *Rhizo11* that were mostly absent in the field (Figure 1), but only the *Rhizo11* changes were statistically significant (DESEQ2: effect size = 0.203, $p = 0.956$; effect size = 0.586, $p = 0.823$; effect size = 5.807, $p = 0.033$, respectively). Among the remaining 15 abundant OTUs, *Entom6*, *Enter7* and *EntAcro2* significantly increased in laboratory colonies as well (DESEQ2: effect size = 3.810, $p = 0.045$; effect size = 2.663, $p = 0.033$; effect size = 4.706, $p = 0.033$, respectively), but these significances also barely passed the 5% threshold, so may not be meaningful because we did not correct for repeated testing. The abundant OTUs *EntAcro1* in *Atta* and *Acromyrmex*, *RhiAcro1* in *Ac. octospinosus* and *T. cornetzi*, *EntAcro10* in *T. zeteki*, *ActAcro1* in *C. costatus*, *Rhizo8* in *C. longiscapus*, and *WolAcro1* in *Acromyrmex* and *Ap. dentigerum* were hardly affected by transfers of colonies from the field to the laboratory (Figure 1, all DESEQ p -values ≥ 0.2). We therefore conclude that, in spite of the suggestive differences between field and laboratory colonies for some of the ant species emanating from Figure 1, few of these represented statistically meaningful shifts in bacterial community.

While we could not recover a general association with alpha diversity of abdominal bacterial communities, the presence/absence of cuticular Actinobacteria did reveal shifts in specific representations of abdominal OTUs. We found that *WolAcro1*, *Entom6*, *Actin20* and *Clost64* were present in significantly higher prevalences in abdominal tissues of ants carrying cuticular Actinobacteria (Figure 3 and Supporting information Table S6; DESEQ2, adjusted p values for *Actin20* $p = 0.01$, for the other three OTUs $p < 0.001$). Two other OTUs were largely specific for ants that lack cuticular Actinobacteria: a Rhizobiales (α -Proteobacteria) OTU (*Rhizo11*) ($p < 0.001$) and a Solirubrobacterales (Actinobacteria) OTU (*Solir27*) ($p = 0.03$). These results suggest that the presence/absence of

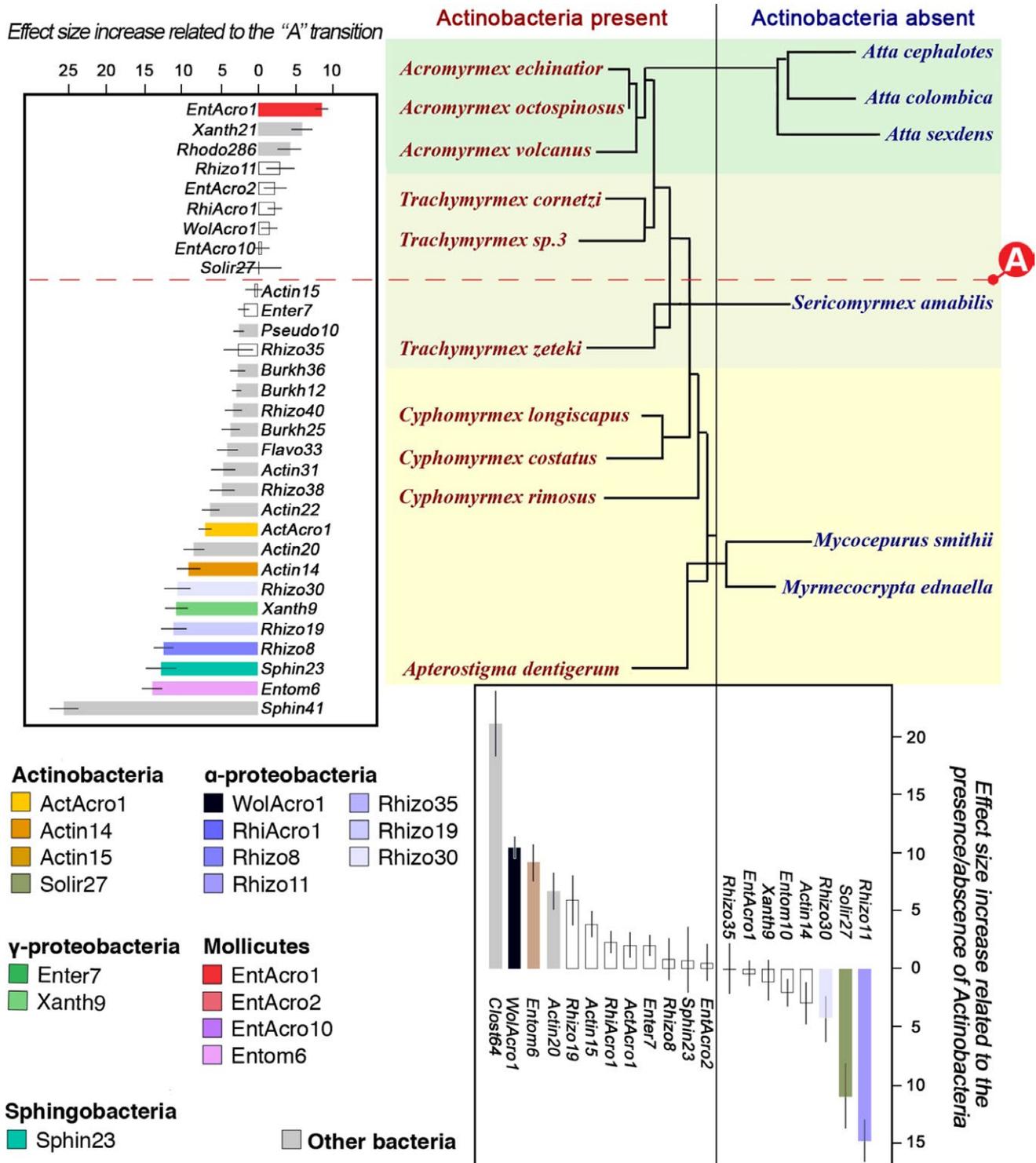


FIGURE 3 Patterns of differential representation of abdominal bacterial OTUs across sixteen Panamanian fungus-growing ant species, based on all 2099 OTUs. The ant phylogeny of Figure 1 is redrawn here in such a way that species with and without visible covers of cuticular Actinobacteria are separated into left and right columns, with the traditional farming transitions highlighted by the same background colour shifts as in Figures 1 and 2 and Supporting information Figure S4, and the "A" shift identified in Figure 2 added as a red dashed horizontal line contrasting with the vertical black line highlighting the presence/absence of cuticular actinobacterial biofilms. DESEQ2 analyses (Supporting information Table S6) showed that both partitions affected the proportional representation of a series of OTUs, of which we plotted all 18 abundant OTUs and the 14 remaining less common OTUs for which differential representation was significant at $p < 0.05$. Bars show effect size differences \pm SE for each OTU as identified by the DESEQ2 analysis (Supporting information Table S6). Bar colours for specific OTUs are the same as in Figure 1 (legend bottom left), and grey bars present the 14 nonabundant OTUs across both histograms, which are not plotted in Figure 1 but for which representations were significantly different in the present analysis. Open white bars represent abundant OTUs whose differential representation was not affected by either of the two focal partitions

cuticular Actinobacteria on the propleural thorax plates of worker ants may encourage the growth of certain abdominal endosymbiont OTUs while inhibiting the growth of others.

Finally, we checked whether overall bacterial abundances in ant abdomens, as measured by qPCR, were affected by the presence/absence of cuticular Actinobacteria or by transfer of colonies to the laboratory. Our analysis based on entire abdomens revealed no association between bacterial titres and either sampling environment (MCMCGLMM: $p = 0.209$; Supporting information Table S7E) or the presence/absence of Actinobacteria ($p = 0.980$; Supporting information Table S7E), nor for the statistical interaction term between these two main predictor variables ($p = 0.513$; Supporting information Table S7E). This was also the case when we used the uncorrected qPCR results from our mixed set of dissected abdomen samples and entire abdomen samples (Supporting information Results S3).

3.4 | Phylogenetic and environmental effects on OTU community composition

We performed beta diversity analyses to evaluate whether there were any discontinuous shifts in the composition of endosymbiotic abdominal communities across the attine phylogeny, and whether any such putative shifts were affected by the presence/absence of cuticular Actinobacteria. At the same time, such analyses allowed us to evaluate whether or not transfer of colonies to a common laboratory environment tends to homogenize bacterial communities, that is, reduce the variance in OTU communities between colonies. Before doing these analyses, we checked whether confounding effects might have affected our beta diversity estimates (Supporting information Results S1). We found evidence for similar PCR method effects (justifying exclusion of the touchdown PCR samples also

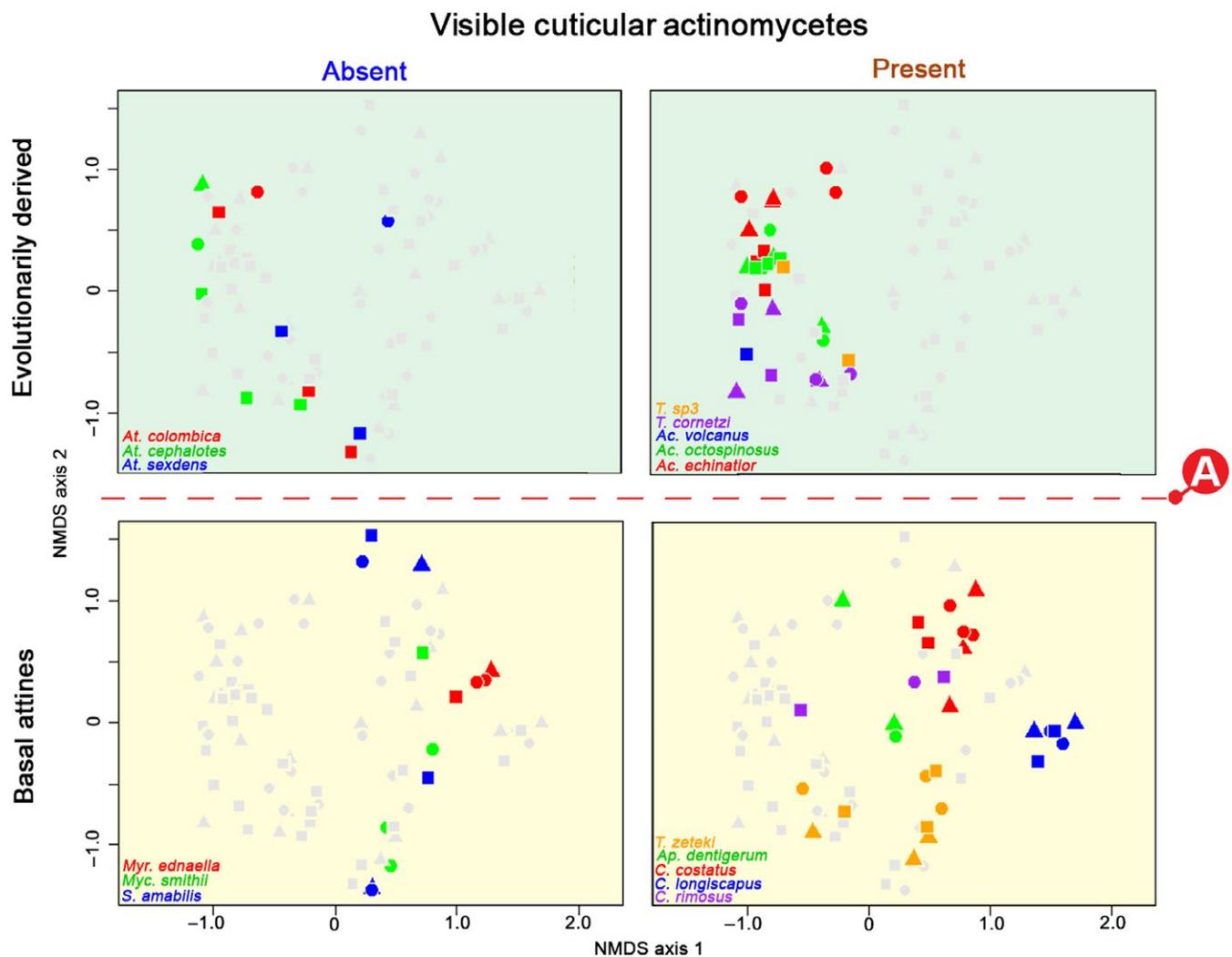


FIGURE 4 Composition of abdominal bacterial communities (beta diversity) partitioned according to phylogeny and presence/absence of cuticular Actinobacteria across 16 species of Panamanian fungus-growing ants, based on 84 colony samples and using only the 18 most abundant OTUs. The ordination is based on nonmetric multidimensional scaling ordination of pairwise Bray–Curtis dissimilarities between all 84 colony samples amplified with standard PCR and had an overall stress factor of 0.208, which implies that the representation of total variation by just two axes was acceptable. The closer two samples are on the ordination plot, the more similar their bacterial communities. The three types of samples for each ant species were as follows: field colonies (circles), short-term laboratory colonies (triangles) and long-term laboratory colonies (squares). The four panels each have all 84 colony samples plotted, with samples from the focal quadrant coloured by species, and the remaining samples in grey

here) and for the number of individuals used for DNA extraction having affected the community of OTUs retrieved. However, we concluded that the latter confounding effect (explaining <9% of the variance in bacterial community composition) was minor relative to the much more pronounced between-species effect that explained almost 40% of the variance (Supporting information Results S1).

PERMANOVA showed that both the phylogenetic transition “A” and the presence of cuticular Actinobacteria had a significant effect on the mean compositions of abdominal bacterial communities (Figure 4) but that the former had a higher impact ($Pseudo-F_{1,78} = 14.69$, partial $R^2 = 0.148$, $p < 0.001$ and $Pseudo-F_{1,78} = 3.747$, partial $R^2 = 0.038$, $p = 0.001$, respectively). There was also an interaction between phylogenetic transition and the presence of Actinobacteria that just fell short of significance ($Pseudo-F_{1,78} = 1.81$, partial $R^2 = 0.018$, $p = 0.082$), while sampling environment had no overall impact ($Pseudo-F_{2,78} = 0.530$, partial $R^2 = 0.011$, $p = 0.927$). As these analyses focused on shifts in bacterial communities rather than on anonymous OTU richness or alpha diversity, the results suggest that transition “A” also carries a signature of change in beta diversity.

These changes in mean bacterial communities were also supported by our linear discriminant analysis (DA) results. Almost all (90.5%) of the colony-level samples could be correctly assigned to above or below this transition based on their bacterial community composition (entropy $R^2 = 0.634$), while the presence/absence of cuticular Actinobacteria could not be deduced from these community compositions (57.2% correctly assigned, $R^2 = 0.101$). Particularly, the evolutionarily derived attine species carrying Actinobacteria had very similar abdominal bacterial communities and could not be readily distinguished in discriminant analysis (58.7% correctly assigned, $R^2 = 0.372$), while all other quadrants of species were more distinct in their bacterial communities (evolutionarily derived ants without Actinobacteria: 66.7%, $R^2 = 0.405$; basal ants without Actinobacteria: 92.9%, $R^2 = 0.593$; basal ants bearing Actinobacteria: 82.8%, $R^2 = 0.805$). These discriminant analysis results did not change in any appreciable way when we used alternative weighted or unweighted UniFrac distance measures, or when we used all 2099 OTUs instead of only the 18 abundant ones (Supporting information Figure S5 and Table S9).

Finally, we used HOMOVA to explicitly evaluate the changes in between-sample variance in bacterial communities for the 18 abundant OTUs across the “A” transition and the possible codependence of such shifts on the presence/absence of cuticular Actinobacteria (Figure 4 and Supporting information Table S8). Using only the phylogenetic contrast, this confirmed that the evolutionarily derived attine ants (beyond transition “A” in Figure 2) had a significant reduction in the variability of their abdominal bacterial communities compared to the basal attines ($B = 1.2494$, $p < 0.001$ for Bray–Curtis distance; $B = 1.805$, $p < 0.001$ for weighted UniFrac distances; $B = 0.988$, $p = 0.018$ for unweighted UniFrac distances; Supporting information Table S8). The first two distance measures also indicated that the transition “A” effect on variability of OTU communities across samples was driven by the

low variance in bacterial communities of the evolutionarily derived attine ants that carry cuticular Actinobacteria ($B = 2.2194$, $p < 0.001$ for Bray–Curtis distances and $B = 2.9458$, $p < 0.001$ for weighted UniFrac distances; Table S8). Using the full data set of 2099 OTUs produced a comparable result and this time the evolutionarily derived attine contrast between species with and without cuticular Actinobacteria was recovered for all three distance measures, in addition to unanimous support for the “A” transition contrast (Supporting information Table S8, Figure S5). Sampling environment was never a significant copredictor in any of the HOMOVA tests.

4 | DISCUSSION

Our study is the first large-scale comparative analysis of abdominal endosymbionts spanning the entire phylogenetic diversity of the ant tribe Attini. After the ancestor of these ants specialized on fungus farming ca. 55–60 MYA, the symbiosis was elaborated in two steps—first by fully domesticating a single lineage of crop fungi which allowed the ants to abandon less specific subsistence farming, and second by developing industrial-scale leaf-cutting farming in tighter co-evolution with increasingly multinucleate and polyploid crop fungi that gradually adapted to decompose more nutritious fresh plant material (De Fine Licht et al., 2013; Kooij et al., 2015; Nygaard et al., 2016; Schultz & Brady, 2008; Shik et al., 2016). The attine ant symbiosis with fungus gardens became significantly enriched by cuticular Actinobacteria, which were acquired shortly after the origin of fungus farming and persisted in some, but not all later-evolving attine genera where their main function appears to be the control of *Escovopsis* infections or other pathogens in fungus gardens (Barke et al., 2010; Currie et al., 2006; Fernandez-Marin et al., 2013; Fernández-Marín, Zimmerman, Nash, Boomsma, & Wcislo, 2009; Fernández-Marín, Zimmerman, Rehner, & Wcislo, 2006; Gerardo et al., 2006; Haeder et al., 2009; Mattoso et al., 2012; Seipke et al., 2011). Our comparative survey of the composition, variation and robustness of the attine microbiomes associated with the gut and associated organs now adds a third layer of intriguing complexity to this symbiotic model system and strongly suggests that these abdominal microbiomes evolved in direct interaction with the attine ant hosts, the fungal cultivars and the presence/absence of actinobacterial microbiomes on the cuticle of the ants. We identified a clear transition in overall abundances of abdominal microbiota in between the domestication of gongylidia-bearing cultivars and the origin of the *Atta* and *Acromyrmex* leaf-cutting ants. This “A” transition (Figure 2), which showed significant contrasts in: (a) Our analyses of beta diversity (Figure 4; Supporting information Figure S5), (b) our OTU-specific analyses (Figure 3) and (c) some of our alpha diversity analyses (Fig. S4) coincides with the colonization of Central/North America by the first attine ant lineage (Branstetter et al., 2017).

4.1 | Less is more when fungus farming increases in scale and specialization

The overall diversity of the abdominal microbiomes across the attine ants is low. Although we obtained thousands of OTUs, only 21 of these were abundant across 107 samples (76 colonies) from 17 Panamanian ant species, a number that was reduced to 18 OTUs in the 84 samples from 16 ant species that we used for most of the analyses (representing >90% of all original reads). These findings are consistent with a previous study showing that more than 97% of the bacterial sequence reads obtained from the abdominal tissues of Panamanian *Acromyrmex* species is captured by just four of these OTUs (Sapountzis et al., 2015). The overall trends that we uncovered were that: (a) Abdominal bacteria have low or even negligible abundances in lower attine ants and their general abundance increases sharply at transition "A" (Figure 2), (b) This shift in abundance was reflected in patterns of beta diversity, indicating a discontinuous change in abdominal community composition, but whether alpha diversity decreased when abdominal bacterial titres increased remained unclear. Our correlative evidence suggested that multiple aspects of abdominal microbiome composition are affected by the presence/absence of cuticular Actinobacteria, particularly for the evolutionarily derived attines beyond transition "A." We concentrated our sampling efforts on a single Panamanian site of ca. 25 km², where it could reasonably be assumed that all ant species were exposed to the same environmental bacteria, so that noise from this source across genera and species of attine ants would be minimal. The extent to which our results are representative for other Latin American regions remains to be demonstrated and we hope that such studies will be pursued in the years to come.

We obtained several lines of evidence indicating that the most important transition in overall bacterial abundance and community composition (beta diversity) of abdominal microbiomes occurred after the origin of higher attine agriculture but well before the origin of industrial-scale leaf-cutting agriculture (Schultz & Brady, 2008). This transition seemed puzzling initially, but turned out to match a recent study showing that the ancestor of the crown group starting with the *T. cornetzi* branch ("A" in Figure 2) colonized the Central/North American subcontinent long before the Isthmus of Panama closed (Branstetter et al., 2017). This finding is intriguing because the founding queens of *T. cornetzi* are very small, which may have facilitated this unique event of long-distance airborne dispersal. These undoubtedly severely bottlenecked colonizers were the first fungus growers in an entire "northern" subcontinent and started an adaptive radiation that ultimately produced the *Acromyrmex* and *Atta* leafcutter ants, which thus appear to have evolved in Central/North America rather than in South America as has previously been assumed (Branstetter et al., 2017). Only much later (presumably when the Isthmus was about to close) did representatives of *Acromyrmex* and *Atta* (whose queens are much heavier) colonize South America. The phylogenomic ancestral state reconstructions by Branstetter et al. (2017) imply that the "A" transition in abdominal microbiomes uncovered in our study coincides with what must have been the most

significant vicariance event in the adaptive radiation of the attine ants. If our results would prove to have more general validity across Latin America, they could imply that obligate crop domestication, that is, the emergence of gongyldia-bearing cultivars, affected gut microbiota less than the subsequent colonization of the Central/North American subcontinent. This is because bacterial species composition in the abdomens of Panamanian *T. zeteki* and *S. amabilis* remained rather similar to those of the lower attine ants included in our study. It is also striking that the common Mollicutes OTU *EntAcro10* was found in all genus-level branches of the attine tree, but that the abundant leaf-cutting ant OTU *EntAcro1* was also found in *T. cornetzi* (Figure 1; see also Sapountzis, Zhukova, Shik, Schiott, & Boomsma, 2018). Also, the α -Proteobacteria (mostly Rhizobiales and *Wolbachia* in *Acromyrmex*) begin to rise to abundance from the *T. cornetzi* lineage onwards only to be reduced to minor significance again in *Atta*.

4.2 | Putative functions of abundant abdominal endosymbionts

As new attine genera emerged over evolutionary time, a few specific OTUs became dominant (Figure 1), suggesting that these eclectic higher attine symbionts came to provide specific mutualistic services when ant fungus farming became more advanced. Rhizobiales are particularly abundant in higher attine guts beyond the "A"-transition, where they are largely restricted to the hindgut (rectum and ileum) in *T. cornetzi*, similar to *Acromyrmex* species where an earlier study recovered both *nifH* sequences and NifH proteins from the hindgut biofilms of *RhiAcro1* bacteria. This suggested that at least some strains encompassed by the *RhiAcro1* OTU are able to retrieve nitrogen for the farming symbiosis (Sapountzis et al., 2015), but alternative functions are also possible (Bradley & Philips, 1977; Donini & Odonnell, 2005; Engel & Moran, 2013; Russell et al., 2009; Sapountzis et al., 2015). The exclusive presence of *RhiAcro1* in Panamanian *Ac. echinator*, *Ac. octospinosus*, *T. cornetzi* and *T. sp. 3* makes it of interest to find out whether similar patterns and putative functions can be found in other *Acromyrmex* species and representatives of the *T. cornetzi* clade that colonized South America, and in *T. septentrionalis* which is the sister lineage of the leaf-cutting ants and occurs only in North America.

The other occasionally abundant α -Proteobacterium associated with the abdominal organs was *Wolbachia*, but this symbiont (*WolAcro1*) was only found in consistent prevalences in *Acromyrmex* species and, to a lesser extent, *Ap. dentigerum* (Figure 1), confirming previous findings (Andersen et al., 2012; Frost, Fernandez-Marin, Smith, & Hughes, 2010; Sapountzis et al., 2015). These bacteria primarily colonize nonreproductive tissues in *Acromyrmex* (Andersen et al., 2012; Frost, Pollock, Smith, & Hughes, 2014) where they can also occur extracellularly in the gut lumen, although it remains unknown how many bacterial cells may occur in this form. Our FISH location evidence suggests that *Wolbachia* cells are almost absent in somatic tissues of *Ap. dentigerum* but more abundant in the ovaries, whereas this appears to be the other way around in *Ac. echinator*

(P. Sapountzis, unpublished observations). This may be consistent with a putative mutualistic function of this symbiont in evolutionarily derived *Acromyrmex* leaf-cutting ants (see Andersen et al. (2012) for a more detailed discussion) that did not evolve in the phylogenetically basal *Apterostigma*, but further work will be needed to clarify this.

The functional significance of the few but occasionally highly abundant Mollicutes OTUs remains enigmatic. It is very unlikely that they are pathogens (Anderson et al., 2012; Funaro et al., 2011; Kautz, Rubin, Russell, & Moreau, 2013; Meirelles et al., 2016; Sapountzis et al., 2015) and rather suggests that the high prevalences of *EntAcro1* in the leaf-cutting ants may be related to actively herbivorous foraging habits (De Fine Licht & Boomsma, 2010) which also implies the ingestion of plant sap and fruit juices (Littledyke & Cherrett, 1976). Their consistent localization in the fat body suggests that beneficial interactions with energy and nutrient decomposition processes are likely (Arrese & Soulages, 2010), particularly because some close relatives of the two most common *EntAcro1* and *EntAcro10* OTUs are associated with other insects with specialized diets where they may be mutualists (Lo, Ku, Chen, Chang, & Kuo, 2013). Detoxification and immune defence functions are also a possibility when these bacteria occur in the Malpighian tubules as is commonly observed (Beyenbach, Skaer, & Dow, 2010; Dow, 2009). A possible connection with chitin monomer degradation has been suggested by Sapountzis et al. (2015), with recent reinforcement from an extensive comparative genomics analysis across the crown group of the same Panamanian attine ants as covered in our present study. That study revealed consistent signatures of positive selection for novel chitinase decomposition functions in ant genomes, likely in response to positive selection for chitin synthases in the fungal cultivars (Nygaard et al., 2016). The chitin metabolism hypothesis is also, albeit indirectly, supported by our 16S-sequencing results showing that several *Chitinophaga* OTUs (order Sphingobacteriales, class Sphingobacteria) occur in the abdomens of some *C. longiscapus* and *Myr. ednaella* workers (Supporting information Table S3). These two species happen to be the only attines where Mollicutes were always sparse (<4% across 107 samples; see Supporting information Table S3), but any such inferences remain provisional and will need explicit validation. We address the functional significance of *EntAcro1* and *EntAcro10* in a parallel study (Sapountzis et al., 2018).

The most abundant actinobacterial OTU associated with the abdomens of attine ants was *ActAcro1*, a *Pseudonocardia* that has previously been found in minor (<1%) prevalences in the abdomens of some Panamanian *Acromyrmex* workers (Sapountzis et al., 2015), where it is primarily an obligate cuticular ectosymbiont producing antibiotics to help control *Escovopsis* infections (Currie, Bot, & Boomsma, 2003). The same OTU now appears to be relatively prevalent in the sparse endosymbiotic microbiomes of lower attine ants (Figure 1; mean prevalence per species 5%–37%) with the exception of *C. longiscapus* where it is very sparse (mean prevalence <0.3%). These numbers changed only slightly when separately considering the abdominal microbiomes of workers in field colonies, because prevalences of *ActAcro1* in the abdomens of lower attines tended to remain similar (6%–36%) after transfer of colonies to the laboratory. The prevalences of *ActAcro1* in *Myr. ednaella* were in fact higher in field colonies (16%–24%) than in laboratory colonies (2%–5%). These

consistent presences of *Pseudonocardia* in lower attine abdomens can therefore not be explained by contamination of the gut after ingesting cuticular *Pseudonocardia*, also because *ActAcro1* was present in lower attines that lack cuticular Actinobacteria (the *Myrmicocrypta* and *Mycocephurus* used in our study), in *Cyphomyrmex* lower attines where cuticular *Pseudonocardia* is absent or rare (Andersen et al., 2013; Innocent et al., unpublished results), and in higher attines such as *T. zeteki* that have cuticular actinobacterial biofilms with other bacterial species than *Pseudonocardia* (Andersen et al., 2013; Sen et al., 2009). This could imply that *ActAcro1* was initially a gut or abdominal-organ symbiont, whose antibiotic functions were later co-opted for external hygienic use in the evolutionarily derived higher attine ants. This hypothesis deserves further testing because it may shed novel light on the ongoing discussion of whether and to what extent *Pseudonocardia* strains co-evolved with the attine ant symbiosis and its *Escovopsis* mycopathogens (Cafaro et al., 2011; de Man et al., 2016; Heine et al., 2018; Holmes et al., 2016; Sen et al., 2009).

Members of γ -Proteobacteria were found in the attine ant abdomens but only in traces and then primarily in the rectum (Figure 2). This finding is of interest because representatives of γ -Proteobacteria have repeatedly been reported as being abundant in the fungus gardens of attine ants (Aylward et al., 2014, 2012; Kellner, Ishak, Linksvayer, & Mueller, 2015; Pinto-Tomás et al., 2009), although it remains unclear whether these were the same OTUs or not. These previously reported γ -Proteobacteria are mostly Enterobacteriaceae with abilities to fix nitrogen in laboratory fungus gardens of *Atta* leaf-cutting ants (Pinto-Tomás et al., 2009). They not only occur abundantly in fungus gardens (Aylward et al., 2014) but also have a substantial presence in the abdominal microbiomes of leaf-cutting ant larvae who only ingest fungal food, suggesting they may have important metabolic roles during development (Zhukova, Sapountzis, Schiøtt, & Boomsma, 2017). However, the scattered pieces of evidence for nitrogen supplementation by bacterial symbionts, suggested for the hindgut of *Acromyrmex* workers (Sapountzis et al., 2015) and shown for *Atta* fungus garden (Pinto-Tomás et al., 2009), are as yet insufficient to understand the bigger picture of nitrogen turnover in attine ants (Shik et al., 2016). Nitrogen supplementation by bacterial symbionts has been suggested to also be important in other ant species with nitrogen limited diets (Russell et al., 2009; van Borm, Buschinger, Boomsma, & Billen, 2002), but more work is needed to resolve when and why during attine ant evolution nitrogen became a limiting factor for mutualistic productivity and whether nitrogen supplementation in the hindgut and fungus garden would tend to complement or exclude each other.

4.3 | Interactions between thoracic cuticular Actinobacteria and abdominal microbiomes

We obtained multiple pieces of correlative evidence suggesting that the presence or absence of cuticular Actinobacteria affects the composition of abdominal microbiomes (Figures 3 and 4; Supporting information Figure S5; Tables S8 and S9), but not their overall abundances which seem to be largely dependent on the phylogenetic position of ant species and genera (Figure 2; Supporting information Results S3).

These effects appear to be complex, to probably interact with the “A” transition, and to be mostly reflected in distributional shifts among rarer OTUs rather than the abundant ones (Figure 3). Given the known production of antibacterials by the *ActAcro1* cuticular *Pseudonocardia* symbiont (Holmes et al., 2016), these interactive effects do not come as a surprise, but they will require functional experiments to assess whether and to what extent specific bacterial OTUs benefit from the presence of cuticular actinobacterial antibiotics or are inhibited.

Our quantitative comparisons of shifts in community composition of abdominal bacteria and the general robustness of these communities across sampling environments showed that ant species that carry cuticular Actinobacteria tend to have more similar abdominal microbiomes than those without, particularly in the evolutionarily derived attine lineages (Figure 4; Supporting information Figure S5). This implies that several attine ant species may have abdominal microbiota that could be actively protected by cuticular antibiotic-producing biofilms against inadvertent colonizers that may compete with “native” abdominal endosymbionts. This hypothesis will also need to be tested experimentally.

4.4 | Caveats and future perspectives

Our comparative 16S-metagenomic study adds significant insights into the complexity of the attine ant fungus farming symbiosis across their phylogenetic tree, but surveys of this kind cannot resolve hidden diversity that may exist between closely related symbionts that share identical or very similar (>97%) 16S sequences (Andersen et al., 2012; Engel, Stepanauskas, & Moran, 2014; Kuo, 2015). They also do not provide direct information on the function of symbiotic OTUs, so the correlative trends and inferences obtained in our present study need to be followed up by more extensive microscopy of bacteria from field-collected attines (see e.g., Sapountzis et al., 2015) and by functional genomic studies of the handful of abundant bacterial symbionts that we identified and mapped onto abdominal tissues and organs after FISH (e.g. Sapountzis et al., 2018). Finally, our survey remained restricted to central Panama and needs to be repeated in South America where extant diversity of attine ants is higher. Comparisons between attine ant communities in Central and South America have become relevant in a novel way after Branstetter et al. (2017) showed (in sharp contrast to previous belief; e.g., Kusnezov, 1963) that most of the crown group of the higher attine ants (including the leaf-cutting ants) evolved in Central/North America. If the discontinuous change in abdominal microbiomes that we identified as the “A”-transition does indeed represent a fundamental reorganization of the abdominal microbiome upon colonization of a new American subcontinent, our results would predict that extant representatives of the *Trachymyrmex*, *Acromyrmex* and *Atta* lineages that independently colonized South America without having evolved there should have retained phylogenetic signatures of their Central/North American origin in their extant abdominal microbiomes, and that those signatures should be similar to what can be found in exclusively North American species of *Trachymyrmex*, *Acromyrmex* and *Atta*.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTION

P.S. performed the experiments. P.S., M.S. and J.J.B. designed the study and wrote the manuscript. P.S. and D.R.N. analysed the data and designed the different types of statistical analysis, D.R.N. helped evaluate the results, conceived and edited part of the figures and contributed to the final stages of the writing. All authors read and approved the final version of the manuscript.

DATA ACCESSIBILITY

Sequences produced for this study are available via the Sequence Reads Archive (<https://www.ncbi.nlm.nih.gov/sra>) under SRA study accessions: SAMN03460089–SAMN03460167 and SAMN03460169–SAMN03460177. OTU tables (Supporting information Table S3) and Results S2 are available on dryad: <https://doi.org/10.5061/dryad.tj30d>.

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SUPPORTING INFORMATION

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Reconstructing the functions of endosymbiotic Mollicutes in fungus-growing ants

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Abstract Mollicutes, a widespread class of bacteria associated with animals and plants, were recently identified as abundant abdominal endosymbionts in healthy workers of attine fungus-growing leaf-cutting ants. We obtained draft genomes of the two most common strains harbored by Panamanian fungus-growing ants. Reconstructions of their functional significance showed that they are independently acquired symbionts, most likely to decompose excess arginine consistent with the farmed fungal cultivars providing this nitrogen-rich amino-acid in variable quantities. Across the attine lineages, the relative abundances of the two Mollicutes strains are associated with the substrate types that foraging workers offer to fungus gardens. One of the symbionts is specific to the leaf-cutting ants and has special genomic machinery to catabolize citrate/glucose into acetate, which appears to deliver direct metabolic energy to the ant workers. Unlike other Mollicutes associated with insect hosts, both attine ant strains have complete phage-defense systems, underlining that they are actively maintained as mutualistic symbionts.

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Introduction

Bacterial endosymbionts, defined here as comprising both intra- and extra-cellular symbionts (*Bourtzis and Miller, 2006*), occur in all eukaryotic lineages and range from parasites to mutualists (*Bourtzis and Miller, 2006; Martin et al., 2017*). Their genomes tend to evolve faster than those of free-living bacteria (*Delaney et al., 2012; Moran et al., 1995*) and they often rely on recombination and horizontal gene transfer when their tissue localizations allow frequent DNA exchange with other bacteria, which tends to purge deleterious mutations when effective population sizes are small (*Naito and Pawlowska, 2016; Takeuchi et al., 2014*). Host-level selection can also induce radical changes in the gene content of endosymbionts (*Wernegreen, 2002*). When they are pathogens such changes can be adaptations to prevail against host defenses or competing bacteria (*Didelot et al., 2016*), as expected from arms races with Red-Queen dynamics (*Mallo et al., 2002; Paterson et al., 2010*). However, when symbionts are mutualists and provide nutritional services, they may become so tightly co-adapted to their hosts that they resemble organelles (*Douglas, 1996; Brinza et al., 2009*). In such cases natural selection is expected to have purged any genes that mediated functions that could be provided more productively by the hosts, a process that has been referred to as Black-Queen dynamics (*Morris et al., 2012*).

The increasing availability of sequenced genomes and accurate molecular phylogenies (e.g. *Leclercq et al., 2014; Gerth et al., 2014*) has allowed a number of intricate endosymbioses between bacteria and arthropods to be understood at functional metabolic levels well beyond qualitative assessments based on 16S ribosomal sequencing. Comparative genomics studies have detected gains and losses of genes or pathways when specialized endosymbionts co-evolve with

eLife digest Bacteria live inside the gut of most creatures. Some are harmful, some beneficial, and some have no clear effects. Studying the genetic material (the genome) of gut bacteria has revealed how they can improve the health, efficiency, and reproductive success of their hosts. For example, studies show that insects with low nutrient diets often benefit from gut bacteria that make vitamins or help them convert food into energy.

Panamanian leafcutter ants live in large colonies and farm fungus for food. They harvest leaves to feed their fungus farms and many are major crop pests in Latin America. How they evolved to be so successful is unclear. Recent studies have shown that huge numbers of bacteria called Mollicutes live in the leafcutter ants' guts. These bacteria do not make the ants sick, so they were thought to be somehow beneficial.

Now, Sapountzis et al. show that the two most common types of Mollicutes found in leafcutter ants evolved to make fungus farming more efficient. The complete genomes of two Mollicutes strains were analyzed and compared to the ones found in other insects. The results showed that both types of Mollicutes can turn excess quantities of the amino acid arginine into a nitrogen-rich fertilizer the ants deposit on their fungal gardens as feces. This helps the ants produce more food. One of the two types can also decompose citrate from plant sap and fruit juice consumed by the ants. This produces acetate, which supplements the ants' fungal diets and provides extra energy.

The unique energy-producing Mollicutes may explain why leafcutter ants evolved larger colonies and sustain higher levels of worker activity than other species of fungus-growing ants. The genome data also showed that both types of Mollicutes have costly defense systems to protect themselves against bacteria-destroying viruses. Many bacteria do not invest in such systems, but the Mollicutes may be able to afford them because their ant hosts provide them with plenty of food. This suggests that both the ants and the Mollicutes benefit from their symbiotic relationship.

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arthropod hosts (Wernegreen, 2002; Didelot et al., 2016; Moran et al., 2008), and have shown that bacterial endosymbionts are particularly useful when their metabolites complement nutrient-poor diets of hosts. Examples are *Buchnera* (γ -Proteobacteria) providing aphids with essential amino acids (Baumann et al., 1995; Shigenobu et al., 2000), *Wolbachia* (α -Proteobacteria) producing vitamin B for *Cimex lectularius* bedbugs (Hosokawa et al., 2010; Nikoh et al., 2014), *Baumannia* and *Sulcia* (γ -Proteobacteria) providing sharpshooters (*Homalodisca coagulata*) with vitamins and amino acids (Wu et al., 2006), *Nardonella* (γ -Proteobacteria) providing beetles with tyrosine required for cuticle formation (Anbutsu et al., 2017), and *Stammera* bacteria allowing leaf beetles to decompose pectin (Salem et al., 2017). A recent comparative analysis confirmed that nutrient supplementation often drives evolution towards host dependence especially when symbionts are vertically transmitted (Fisher et al., 2017).

The social insects with superorganismal colonies, characterized by permanent physiologically and morphologically differentiated castes, appear particularly amenable for hosting specialized bacterial symbionts. Previous studies have documented amino acid provisioning by *Blochmannia* (γ -Proteobacteria) hosted by *Camponotus* carpenter ants (Feldhaar et al., 2007), and suggested that bacterial symbionts provision *Cephalotes* turtle ants with essential amino acids (Hu et al., 2018) and *Cardiocondyla* ants with useful intermediate metabolites (Klein et al., 2016). Honeybees were further suggested to rely on several specialized gut bacteria for carbohydrate breakdown of ingested pollen and nectar (Engel et al., 2012) and fungus-growing termites were discovered to have caste-specific microbiomes depending on whether individuals ingest plant material (mainly decaying wood) or only farmed fungus (Poulsen et al., 2014). Finally, both bees and termites rely on gut microbes to provide them with acetate that can cover up to 100% of their metabolic needs (Odelson and Breznak, 1983; Zheng et al., 2017).

The leaf-cutting ants are the crown group of the attine fungus-growing ants, a monophyletic tribe that evolved 55–60 MYA when their ancestor switched from a hunter-gatherer lifestyle to an exclusive fungal diet (Nygaard et al., 2016; Branstetter et al., 2017). The evolutionarily derived attine lineages rear fully domesticated and co-adapted fungal cultivars that provide the ant farmers with

specialized hyphal tips (gongylidia) containing mostly carbohydrates and lipids that the workers harvest and digest (*De Fine Licht et al., 2014; Quinlan and Cherrett, 1979*). The ant brood is completely dependent on the ingestion of fungal biomass (*Hölldobler and Wilson, 1990*), but workers may ingest and assimilate liquids as well (*Littledyke and Cherrett, 1976; Shik et al., 2018*). However, similar to other ants, they cannot ingest solid plant or animal fragments that they collect to provision their fungus gardens because a sieve in the infrabuccal cavity filters out any particles in excess of ca. 100 μm (*Mueller et al., 2001*). This obligate reciprocity between cultivation and nutrition facilitated further innovations in the terminal clade of *Acromyrmex* and *Atta* leaf-cutting ants, which evolved 15-20 MYA (*Nygaard et al., 2016; Branstetter et al., 2017*). These two genera obtained functionally polyploid cultivars (*Kooij et al., 2015*), adopted multiple queen-mating so their colonies became genetic chimeras (*Villesen et al., 2002*), and became herbivores with massive ecological footprints in Latin America (*Schultz and Brady, 2008; Mehdiabadi and Schultz, 2010; Schiøtt et al., 2010; Leal et al., 2014; Shik et al., 2014*).

Previous studies have shown that *Acromyrmex* and *Atta* leaf-cutting ants harbor low-diversity microbiomes, which include *Wolbachia* (only in *Acromyrmex*), Mollicutes and hindgut *Rhizobiales* (*Van Borm et al., 2002; Andersen et al., 2012; Sapountzis et al., 2015; Meirelles et al., 2016*), symbionts that were inferred to possibly complement the nitrogen-poor diets of *Acromyrmex* leaf-cutting ants (*Sapountzis et al., 2015*). Depending on the actual species studied, Mollicutes – tiny bacteria that lack a cell-wall – can often be found as abundant endosymbionts in up to 100% of leaf-cutting ant colonies (*Sapountzis et al., 2015; Meirelles et al., 2016; Zhukova et al., 2017*), but the absence of in-depth genomic studies has precluded more than speculation about their putative roles as either parasites (*Meirelles et al., 2016*) or mutualists (*Sapountzis et al., 2015*).

To clarify the functional metabolic properties of attine-associated Mollicutes, we mapped the abundances of the two most common strains, *EntAcro1* and *EntAcro10* (cf. *Sapountzis et al., 2015*), in thirteen Panamanian fungus-growing ant species and compared these abundances with the typical spectrum of forage-material that different fungus-farming ants collect and use as compost to manure their fungus-gardens (*Kooij et al., 2014a; Leal and Oliveira, 2000; Shik et al., 2016*). The Panamanian fauna of attine ants encompasses nine of the 17 known genera, including the three most basal genera (*Apterostigma*, *Mycocepurus* and *Myrmicocrypta*), two other basal genera (*Cyphomyrmex* and *Mycetophylax*) being more closely related to the *Trachymyrmex* and *Sericomyrmex* lineages that arose and diversified while rearing gongylidia-bearing cultivars, and finally the *Atta* and *Acromyrmex* leaf-cutting ants who came to practice fungus-farming at an ‘industrial’ scale (*Branstetter et al., 2017; Schultz and Brady, 2008; Mueller et al., 1998*). To explore nutritional mechanisms underlying putative mutualistic functions of these bacteria, and their association with changes in scale of farming over evolutionary time, we isolated and sequenced the *EntAcro1* and *EntAcro10* symbionts (*Sapountzis et al., 2015; Zhukova et al., 2017*). We subsequently compared their draft genomes with ten published genomes of Mollicutes associated with insect hosts having specialized diets, as well as with several other Mollicutes genome-sequences to assess enrichments and losses of gene categories and metabolic pathways.

For *EntAcro1*, where the genomic data suggested the most advanced mutualistic functions, we measured expression levels of bacterial transporter genes related to the decomposition of plant-derived compounds and ant genes related to the uptake of exogenous acetate, an end-product of Mollicutes’ anaerobic metabolism. Our genome comparisons also allowed us to evaluate arginine decomposition functions and defense mechanisms against bacteriophage attack, assuming that: i) variable food-borne arginine supplementation by the fungal cultivar (*Nygaard et al., 2016; Nygaard et al., 2011*) may have offered a niche to both Mollicutes symbionts to convergently evolve similar mutualistic interactions with attine ants, and ii) the abundant and specific bacteriophage sequences that we obtained in the libraries of the *EntAcro1* symbiont indicate that these bacteria have been under selection to maintain costly defenses because extracellular life in the gut lumen likely exposes them to frequent phage encounters.

Results

Metagenome sequencing and phylogenomics

De-novo assembly, annotation and phylogenetic binning of contigs generated from *Ac. echinator* fecal fluid and *Ap. dentigerum* fat body produced a single bin of contigs for each symbiont, confirming them to be valid bacterial species that we will henceforth refer to as *EntAcro1A* and *EntAcro10A*; **Supplementary file 1A**). The predicted coding sequences gave top matches with previously sequenced Mollicutes strains (**Supplementary file 1B**) and each of the bins had a single rRNA operon organized as 16S, 5S, 23S (**Figure 1—figure supplement 1**), similar to closely related *Spiroplasma* (Ku et al., 2013; Lo et al., 2013; Chang et al., 2014) and identical to OTUs in a previous 16S phylogeny (Sapountzis et al., 2015). Additional bins (B; see **Supplementary file 1B and 1C**) did not contain relevant bacterial sequences and were not considered further, similar to the *EntAcro1C* bin that contained bacteriophage sequences with similarity to members of the Gokushovirinae sub-family (*Microviridae* family), a phage lineage known to infect *Spiroplasma* (Chipman et al., 1998; **Supplementary file 1**). Further analyses of the annotated coding sequences confirmed that *EntAcro1A* and *EntAcro10A* represented discrete draft genomes of *EntAcro1* and *EntAcro10* with no or very few missing genes. These genomes had 758 and 776 coding sequences, respectively, and genome sizes of less than 0.9 Kb based on the annotation features (**Supplementary file 1A; Figure 1—figure supplement 1**).

A total of 59 published Mollicutes genomes were used for phylogenomic reconstructions after their predicted proteins gave clear matches to the *EntAcro1* and *EntAcro10* amino acid sequences (**Supplementary file 1B**). This produced nearly identical trees after maximum likelihood (**Figure 1**) and Bayesian analysis of nucleotide and amino acid sequences (**Supplementary file 2; Figure 1—figure supplement 2**) and revealed that *EntAcro1* and *EntAcro10* belong to the Entomoplasmatales group, confirming earlier 16S assignments (Sapountzis et al., 2015). This clade contains *Spiroplasma* and *Mesoplasma* bacteria associated with insects and plants and *Mycoplasma* bacteria known to be mammalian pathogens. Sister-group relationships showed that *EntAcro1* and *EntAcro10* are not closely related and thus likely to have been independently acquired as attine ant symbionts. *EntAcro10* is a relatively basal *Spiroplasma*-like species, a genus with pathogenic, mutualistic and yet unknown interactions with mostly arthropod hosts. However, *EntAcro1* is sister to the *Mesoplasma/Mycoplasma* clade and relatively similar to one of the very few Entomoplasmatales known to be associated with plant hosts in a clade that otherwise consists of vertebrate pathogens. The more distant sister clades are also predominantly pathogenic.

Substrate utilization and reconstruction of metabolic pathways

We restricted our comparative evaluations and hypotheses testing to the *Spiroplasma* and *Mesoplasma* symbionts associated with insect hosts (the attine symbionts highlighted in dark yellow in **Figure 1** and the ones in between) and plants (*M. florum*). Functional annotations (eggNOG database) showed that strains clustered primarily based on metabolic genes and secondarily according to shared identity for informational genes (transcription, translation and recombination/repair processes), which both correlated with host associations (**Figure 1—figure supplement 3; Figure 1—figure supplement 3—source data 1**). Functional gene-similarities were confirmed by Mantel tests showing that the Euclidean dissimilarity matrix of orthogroups was more strongly associated with phylogenomic distances between insect hosts ($r^2 = 0.298$, $p=0.036$) than with phylogenomic distances between bacterial species (**Figure 1**; $r^2 = 0.201$, $p=0.133$), suggesting that many genes that adapt Entomoplasmatales symbionts to their hosts have been horizontally acquired. Metabolic reconstructions (KEGG) further suggested that all Entomoplasmatales are facultative anaerobes, because they lack the genes encoding TCA cycle enzymes and are thus universally incapable of oxidative phosphorylation. However, *EntAcro1* likely lost its aerobic abilities completely because pyruvate dehydrogenase genes are also missing. Differences in metabolism between the two attine symbionts and other insect-associated *Spiroplasma/Mesoplasma* strains were primarily found in pathways mediating catabolism of glycerol, dihydroxyacetone (DHA), citrate, arginine, and N-Acetylglucosamine (GlcNAc) (**Figure 2 and Supplementary file 3**).

Metabolic pathway reconstructions were consistent with *EntAcro10* being a less specifically adapted symbiont than *EntAcro1* (**Figure 2**). Inferences of this kind, based on direct similarity

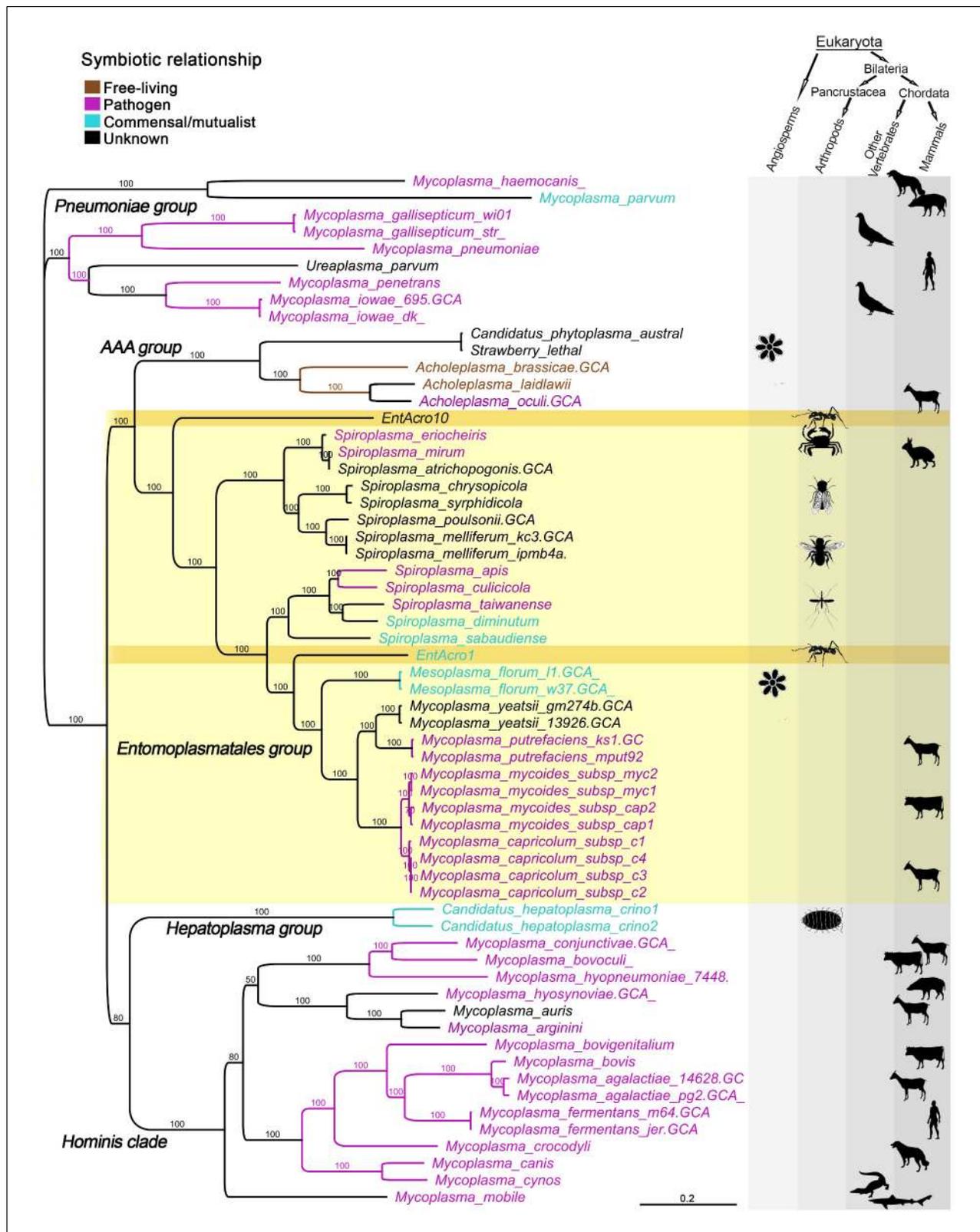


Figure 1. Unrooted phylogeny illustrating the evolutionary relationships and host associations of 59 strains of the bacterial class Mollicutes for which sequenced genomes were available. The Maximum likelihood tree was constructed using a concatenated amino acid alignment of 65 single-copy orthologs (**Supplementary file 2**) giving 100% bootstrap support for almost all branches. Color-coded Latin names specify different types of symbiotic associations (top left, **Supplementary file 4**) and host associations are illustrated by images in the right-hand columns. The light yellow background

Figure 1 continued on next page

Figure 1 continued

highlights all representatives of the Entomoplasmatales and the two ant symbionts *EntAcro1* and *EntAcro10* are marked with a dark yellow background. Black bold-faced text towards the left refers to subclades of Mollicutes identified in a previous phylogenomics study (Leclercq et al., 2014).

DOI: <https://doi.org/10.7554/eLife.39209.003>

The following source data and figure supplements are available for figure 1:

Figure supplement 1. Overview of the assembled draft genome contigs of *EntAcro1A* (top) and *EntAcro10A* (bottom).

DOI: <https://doi.org/10.7554/eLife.39209.004>

Figure supplement 2. Alternative unrooted phylogenies based on the concatenated nucleotide and protein alignments of the 65 single-copy orthologs showing the phylogenetic relationships among 59 Mollicutes strains with sequenced genomes.

DOI: <https://doi.org/10.7554/eLife.39209.005>

Figure supplement 3. Principal Components Analyses (PCA) visualizing gene content similarities of *EntAcro1*, *EntAcro10* and the eleven most closely related Mollicutes genomes.

DOI: <https://doi.org/10.7554/eLife.39209.006>

Figure supplement 3—source data 1. Functional annotation results for the 14 closely related insect-associated Mollicutes genomes using the bacterial eggNOG (bactNOG) database.

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between bacterial genomic databases, may not be fully accurate because gene-families encoding metabolic transporters evolve rather rapidly so the actual transported substrates may no longer be identical. However, the draft genomes that we obtained were sufficiently complete to provide reasonable confidence for reconstruction of operational metabolic transporters through the plasma membrane, the associated metabolic pathways inside the bacterial cells, and the metabolic end-/by-products involved (**Figure 2**). We found that *EntAcro10* can utilize glycerol from ant host cells and monosaccharides which are likely derived from fungus-garden metabolites or juices (glucose/fructose) ingested during foraging (**Figure 2**). We also found an arginine transporter and metabolic genes indicating that *EntAcro10* can decompose arginine. This finding is of interest because the attine ants lost the ability to synthesize this nitrogen-rich amino acid (highest nitrogen to carbon ratio of all amino acids) when fungus-farming evolved, so they obtain arginine from the fungal cultivar and potentially also from ingested fruit juice and plant sap in the herbivorous crown-group leaf-cutting ants (Kooij et al., 2014a; Winter et al., 2015).

The evolutionarily derived *EntAcro1* symbiont had all the transporters and pathways identified for *EntAcro10* but also novel ones that would appear to be particularly adaptive for an abundant extracellular endosymbiont in the gut lumen (Sapountzis et al., 2015) of leaf-cutting ants. First, it has a GlcNAc transporter likely importing chitin monomers, a relatively common metabolic pathway in Mollicutes found in 85 of the 177 genomes that we considered. The possession of a chitin importer is relevant because chitin is one of the most abundant compounds in the ants' fungal diet (**Figure 2**), particularly in the leaf-cutting ants whose cultivars have modified and likely thicker cell walls than the cultivars of phylogenetically more basal attine ants (Nygaard et al., 2016). Second, *EntAcro1* has a rather unique citrate transporter indicative of a rare catabolic pathway observed in only four of the 177 available Mollicutes genomes; **Figure 2—figure supplement 1**). Further examination of the five or six citrate utilization genes involved in this pathway (*citS*, *citC*, *citD*, *citE*, *citF* and potentially *citG*; see **Figure 2—figure supplement 1**) showed that all these genes are extremely rare across the Mollicutes genomes and present similarities to genes from bacterial classes outside the Mollicutes (e.g. Firmicutes and Clostridia) suggesting they were horizontally obtained (**Figure 2—figure supplement 1**). Anaerobic citrate fermentation, or co-fermentation of glucose/citrate, using the *citS-citF* operon will produce acetate (Pudlik and Lolkema, 2011; Starrenburg and Hugenholtz, 1991), which is likely imported by eukaryote cells to fuel metabolism (**Figure 2—figure supplement 2**) or stored in the fat body cells (**Figure 2—figure supplement 3**). The citrate pathway thus appears to reflect that leaf-cutting ants can utilize the citrate metabolite that they are known to ingest in substantial quantities as plant sap when cutting fresh leaves (Littleddyke and Cherrett, 1976) and in the form of other juices when drinking from freshly fallen fruit (De Fine Licht and Boomsma, 2010; Evison and Ratnieks, 2007).

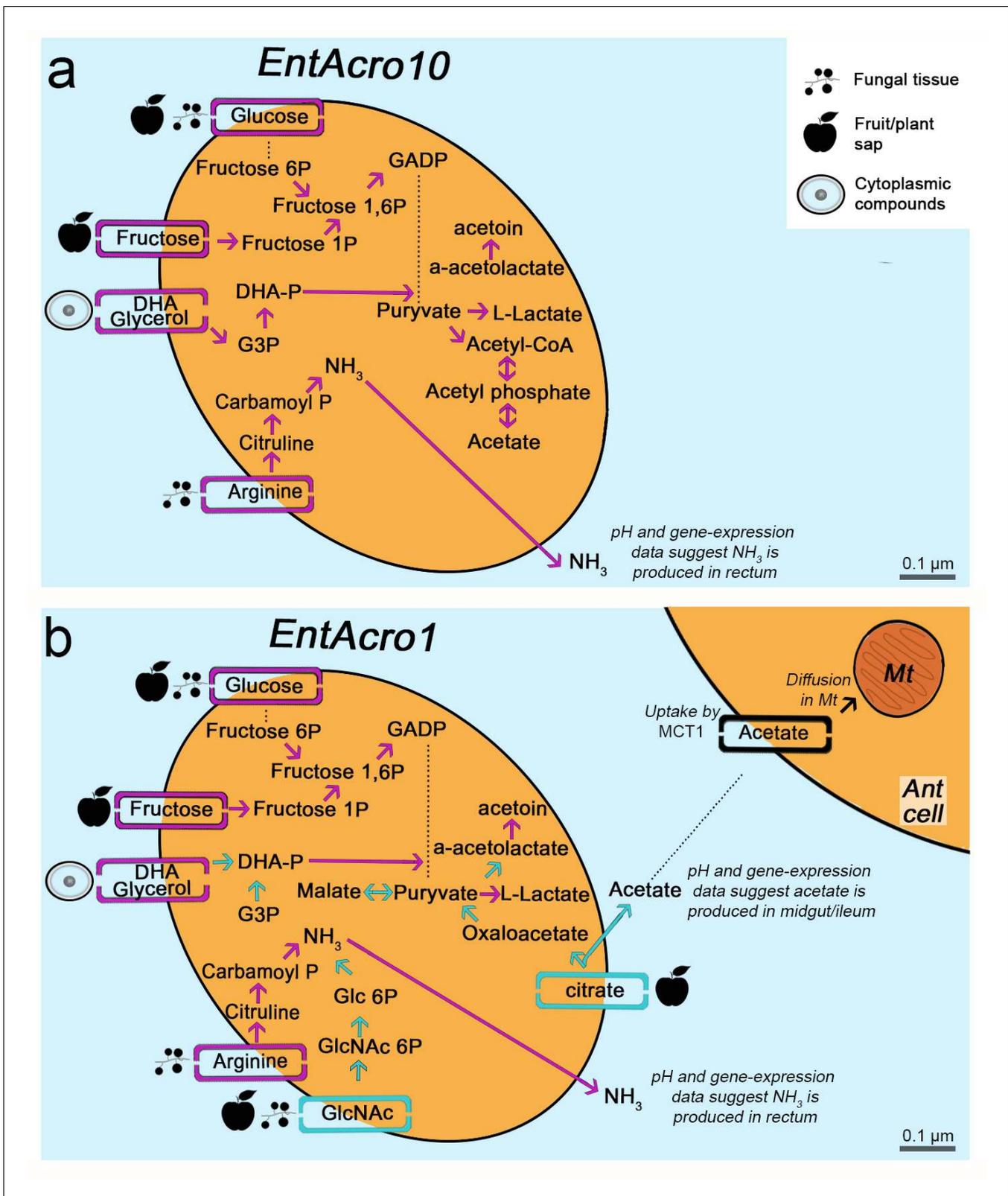


Figure 2. Morphological and metabolic characteristics of the *EntAcro10* and *EntAcro1* symbionts of attine fungus-growing ants. (a) Schematic *EntAcro10* cell with pink arrows representing KEGG pathway reconstructions based on intact genes coding for proteins metabolizing specific compounds and pink rectangles crossing the plasma membrane representing predicted transporter genes that import metabolites from the ant gut lumen where fungal biomass and imbibed fluids are digested (cf symbols next to the transporter rectangles). GlcNAc is present in fungal tissues
 Figure 2 continued on next page

Figure 2 continued

(Pérez and Ribas, 2013) or plant material that may have been decomposed by the fungal cultivar, citrate and glucose/fructose are present in fruit juice and possibly plant sap that ants drink (Liu et al., 2012; Medlicott and Thompson, 1985), arginine is mainly provided by the ingested fungal tissues or juices from leaves and fruits (Nygaard et al., 2016; De Fine Licht et al., 2014; Nygaard et al., 2011; Winter et al., 2015), and glycerol is commonly available in the cytoplasm of eukaryote cells (Grubmüller et al., 2014; Joseph et al., 2008; Monniot et al., 2012; Sun et al., 2003). Dotted lines between metabolic products represent intermediate genes and other metabolic products not shown. Arginine metabolism produces NH_3 , which will be excreted and help the bacteria survive the acidic conditions (Figure 4—figure supplement 1) to end up in the fecal droplets used to manure the ants fungus-garden. The bacterial cell shape and the estimated size (scale bars) were obtained using TEM data from a previous study (Sapountzis et al., 2015). (b) Schematic representation of the *EntAcro1* cell with the functional KEGG pathway reconstructions and predicted transporters inferred from the *EntAcro1* genome that are identical to *EntAcro10* in panel 'a' as pink arrows. Unique additional pathways and transporters only found in *EntAcro1* are drawn in green, and include the anaerobic citrate fermentation pathway, which produces acetate that can be taken up by ant cells, and the GlcNAC pathway that results in byproducts that can enter glycolysis in the bacterial cells.

DOI: <https://doi.org/10.7554/eLife.39209.008>

The following figure supplements are available for figure 2:

Figure supplement 1. Citrate utilization genes in Mollicutes.

DOI: <https://doi.org/10.7554/eLife.39209.009>

Figure supplement 2. Synopsis of the fate of extracellular acetate (on the left) and citrate (on the right) when they enter a eukaryotic cell, based on the available literature.

DOI: <https://doi.org/10.7554/eLife.39209.010>

Figure supplement 3. Schematic representation of two ant fat body cells and the process of storing acetate in the lipids of the fat body cells.

DOI: <https://doi.org/10.7554/eLife.39209.011>

Resource acquisition, gene expression and inferred Mollicutes functions

Screening *EntAcro* abundances in worker bodies across the Panamanian attine ants showed distinct patterns of prevalence. *EntAcro10* was present in most attine species investigated such that there were no significant differences across the entire set of 13 species (planned contrasts, $z = -0.62$, $p=1.00$). However, *EntAcro1* was almost exclusively found in the leaf-cutting ants (planned contrasts, $z = 2.88$, $p=0.016$) with their closest Panamanian relative *T. cornetzi* and yeast-growing *C. rimosus* as the only (partial) exceptions. At the same time *EntAcro10* abundances in leaf-cutting ants were lower than *EntAcro1* abundances albeit only marginally so (planned contrasts, $z = -2.56$, $p=0.045$) (Figure 3A; Figure 3—source data 1). This pattern implies that the appearance of *EntAcro1* is correlated with changes in the spectrum of substrates that the farming ants provide to their fungus gardens (Figure 3B; Figure 3—source data 2), with fresh leaf, fruit and flower provisioning dominating in the leaf-cutting ants and the phylogenetically more basal attines collecting primarily detritus-based substrates such as insect frass and wood chips (Figure 3—figure supplement 1). These differences were variably (non)significant per forage category (Figure 3—figure supplement 1), but bacterial abundances and foraging preferences generally covaried for *EntAcro1* (Mantel test; $r^2 = 0.447$, $p=0.012$) but not for *EntAcro10* (Mantel test; $r^2 = -0.127$, $p=0.882$). These results suggest that the derived metabolic pathways of *EntAcro1* (Figure 2) may have been associated with the expansion of the scale of fungus farming and the adoption of functional herbivory in the leaf-cutting ants.

The abundances of *EntAcro1* bacteria are known to be highly variable, between lab and field colonies, between replicate colonies that seem otherwise fully comparable, and between nestmate workers of both *Atta* and *Acromyrmex* leaf-cutting ants (Sapountzis et al., 2015; Zhukova et al., 2017). To evaluate the potential mutualistic benefit of acetate production by *EntAcro1* (Figure 2—figure supplement 2), we experimentally manipulated *EntAcro1* abundances in lab colonies of *Ac. echinator* and measured rates of acetate uptake in the same ants. We compared ants maintained on their normal fungus garden diet with ants on a sugar/citrate replacement diet with or without antibiotics, known from pilot experiments to remove most Mollicutes from the guts and associated organ systems. We quantified *EntAcro1* abundances by measuring the number of transcripts of the bacterial housekeeping gene *ftsZ* and the rates of acetate uptake by ant host cells by measuring the expression of *MCT1* (Figure 4; Figure 4—source data 1), a gene encoding a plasma membrane protein that imports acetate in eukaryotic cells; Figure 2—figure supplement 2). We found that these variables were positively correlated ($\rho = 0.57$, $p<0.001$), suggesting that acetate production by *EntAcro1* boosts acetate uptake by the ants who likely convert acetate directly into ATP (Figure 2—figure supplement 2). This inference is somewhat tentative because tetracycline can impair

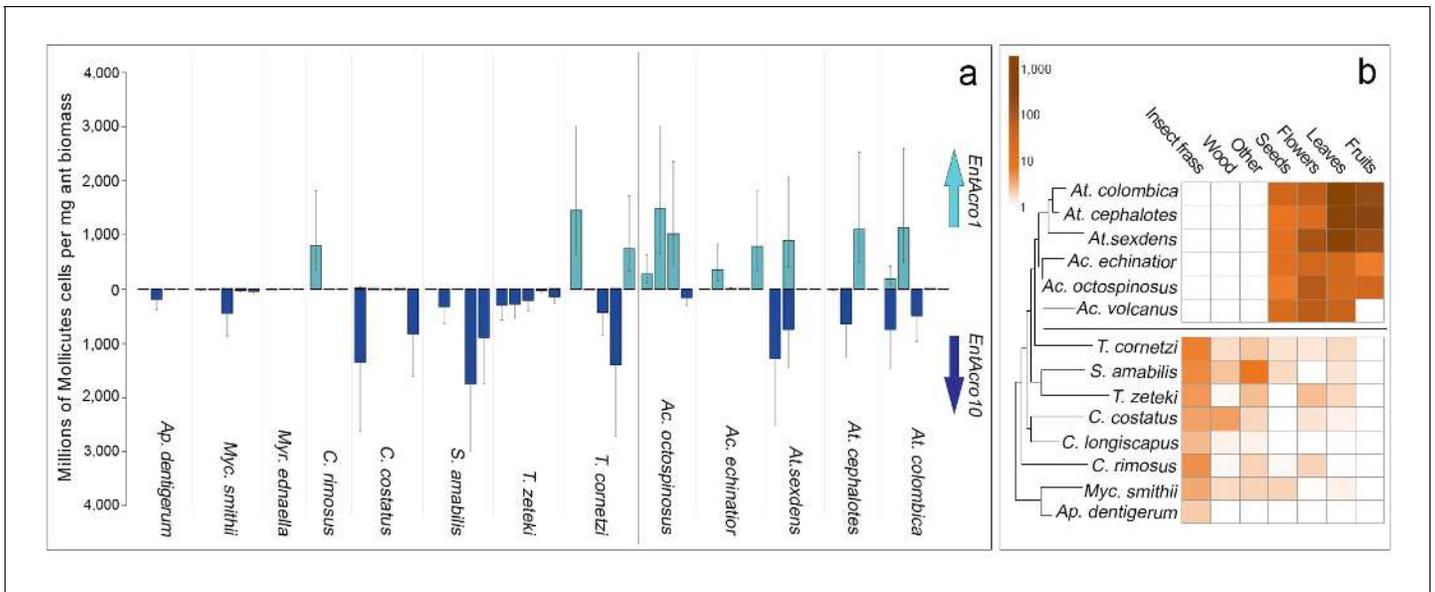


Figure 3. Absolute abundances of *EntAcro1* and *EntAcro10* and foraging substrate preferences across thirteen species of Panamanian fungus-growing ants spanning the entire attine ant phylogenetic tree. (a) Millions of Mollicutes cells per mg of ant worker biomass (means \pm 95% CI based on two technical replicates) across colonies for *EntAcro1* (light blue upward arrow) and *EntAcro10* (dark blue downward arrow). The vertical line between *Ac. octospinosus* and *T. cornetzi* separates the leaf-cutting ants (right) from the non-leafcutting ants (left). (b) Foraging substrate preferences among Panamanian attine ant species presented as mean frequency heat-maps of substrate categories collected per hour for five leaf-cutting ant species (Kooij et al., 2014a) and seven non-leaf-cutting ant species (this study). The tree has been modified from (Branstetter et al., 2017; Schultz and Brady, 2008) and the horizontal line separates the leaf-cutting ants (top) which forage mostly on fresh plant material, from the more basal attine ants (bottom) which forage mostly on detritus based material (Figure 3—figure supplement 1).

DOI: <https://doi.org/10.7554/eLife.39209.012>

The following source data and figure supplement are available for figure 3:

Source data 1. QPCR data for the absolute quantification of *EntAcro* strains in fungus-growing ants.

DOI: <https://doi.org/10.7554/eLife.39209.014>

Source data 2. Merged ant foraging data used for our analyses of data originating from Sapountzis et al. (2015) and the present study.

DOI: <https://doi.org/10.7554/eLife.39209.015>

Figure supplement 1. Principal Components Analysis (PCA) capturing the covariances between attine ant species (colored dots) and foraging substrate preferences (colored arrows) across the Panamanian attine ant hosts of *EntAcro1* and *EntAcro10* endosymbionts (data from Figure 3B, Figure 3—source data 2).

DOI: <https://doi.org/10.7554/eLife.39209.013>

mitochondrial function (Moullan et al., 2015) and thus overall metabolic functionality, a confounder that could have been measured by tracking a specific mitochondrial protein. The fact that the antibiotics data point in Figure 4 directly extended the trend obtained from the treatments without antibiotics (sugar/citrate diet) and the control (fungal diet) suggests that this confounding effect has been minor but further work will be needed to validate this result. Conversion of citrate to acetate by the *EntAcro1* symbiont would be consistent with the general observation that leaf-cutting ants sustain much higher levels of worker activity, both inside nests and while foraging, than phylogenetically more basal attine ants (Kooij et al., 2014a).

To better understand the function of the arginine processing genes (Figure 2), we returned to our comparative genomic data for the attine symbionts *EntAcro1* and *EntAcro10* and the ten *Spiroplasma* species associated with other insects (Figure 5). The observation (Figure 4—figure supplement 1; Figure 4—source data 1) that the arginine transporter of *EntAcro1* was most highly expressed in the hindgut lumen suggests that *EntAcro1* cells may need to decompose arginine in exceedingly low pH conditions (≤ 4). This physiological tolerance may represent a fine-tuned mutualistic service, as became clear when we evaluated patterns of gene expression of all four transporters mediating catabolism of key resources in the gut system and associated ant tissues. The predicted

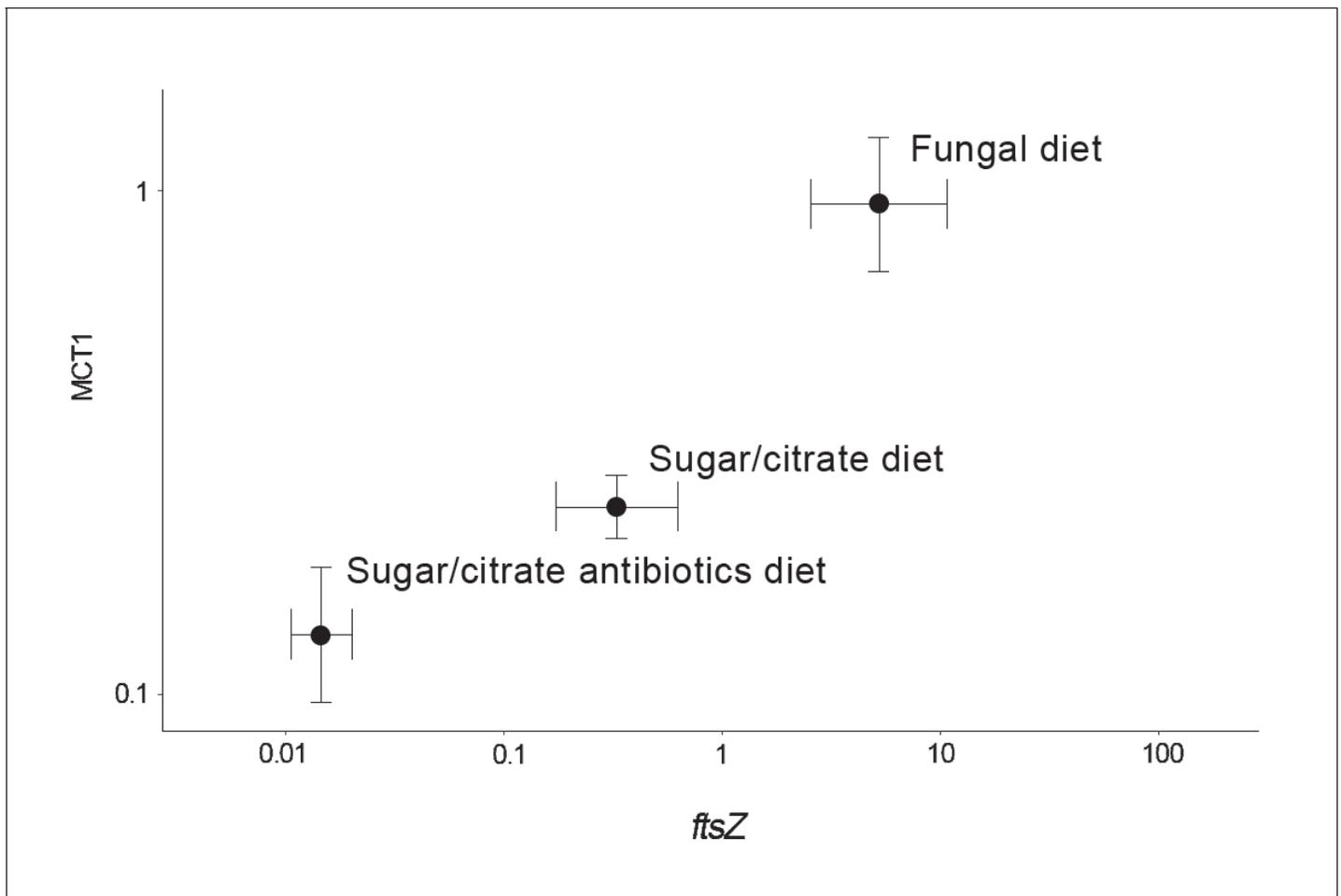


Figure 4. Correlation between the abundance of the *EntAcro1* symbiont (expression of the *EntAcro1* housekeeping gene *ftsZ*) and the activity of the predicted plasma-membrane Monocarboxylate Transporter-1 gene (*MCT1*) in the midgut and fat body tissues of *Ac. echinator* workers. Both axes are logarithmic and express numbers of transcripts normalized relative to the expression of the housekeeping gene *rp17*. Control ants were provided with their natural fungal diet, while manipulated nestmates were kept for seven days without a fungus garden but with access to a glucose/citrate solution with or without antibiotics. Values are means (\pm SEs) of 20 pooled workers replicated twice (technical replicates) across four colonies. The correlation between the two transcript abundances was significant, with the first component of a PCA analysis explaining 91% of the variation. A non-parametric Spearman correlation analysis produced a similarly significant result ($\rho = 0.57$, $p < 0.001$), but correlation tests between *ftsZ* transcripts and the expression of the other MCT-like genes present in the ant genome did not produce significant correlations (**Supplementary file 6**).

DOI: <https://doi.org/10.7554/eLife.39209.016>

The following source data and figure supplements are available for figure 4:

Source data 1. Expression data for the monocarboxylate transporter genes examined in our study.

DOI: <https://doi.org/10.7554/eLife.39209.019>

Figure supplement 1. Differential expression and importance of the four substrate utilization transporter genes of *EntAcro1* that we analyzed.

DOI: <https://doi.org/10.7554/eLife.39209.017>

Figure supplement 1—source data 1. Expression data for the bacterial transporter genes examined in our study.

DOI: <https://doi.org/10.7554/eLife.39209.018>

transporters of arginine, citrate, GlcNAc and glycerol or DHA (**Figure 4—figure supplement 1; Figure 4—source data 1**) were expressed throughout the guts and associated organs of *Ac. echinator* workers, but their expression levels differed across abdominal tissues possibly in response to a steep gradient from pH seven in the midgut and fat body cells where the glycerol transporter is highly expressed, via pH five in the ileum and pH four in the rectum (**Erthal et al., 2004**) where the arginine transporter is highly expressed (**Figure 4—figure supplement 1; Figure 4—source data 1**). Earlier work has indicated that utilization of the *citS-citF* operon is most efficient at or just above pH 5.5

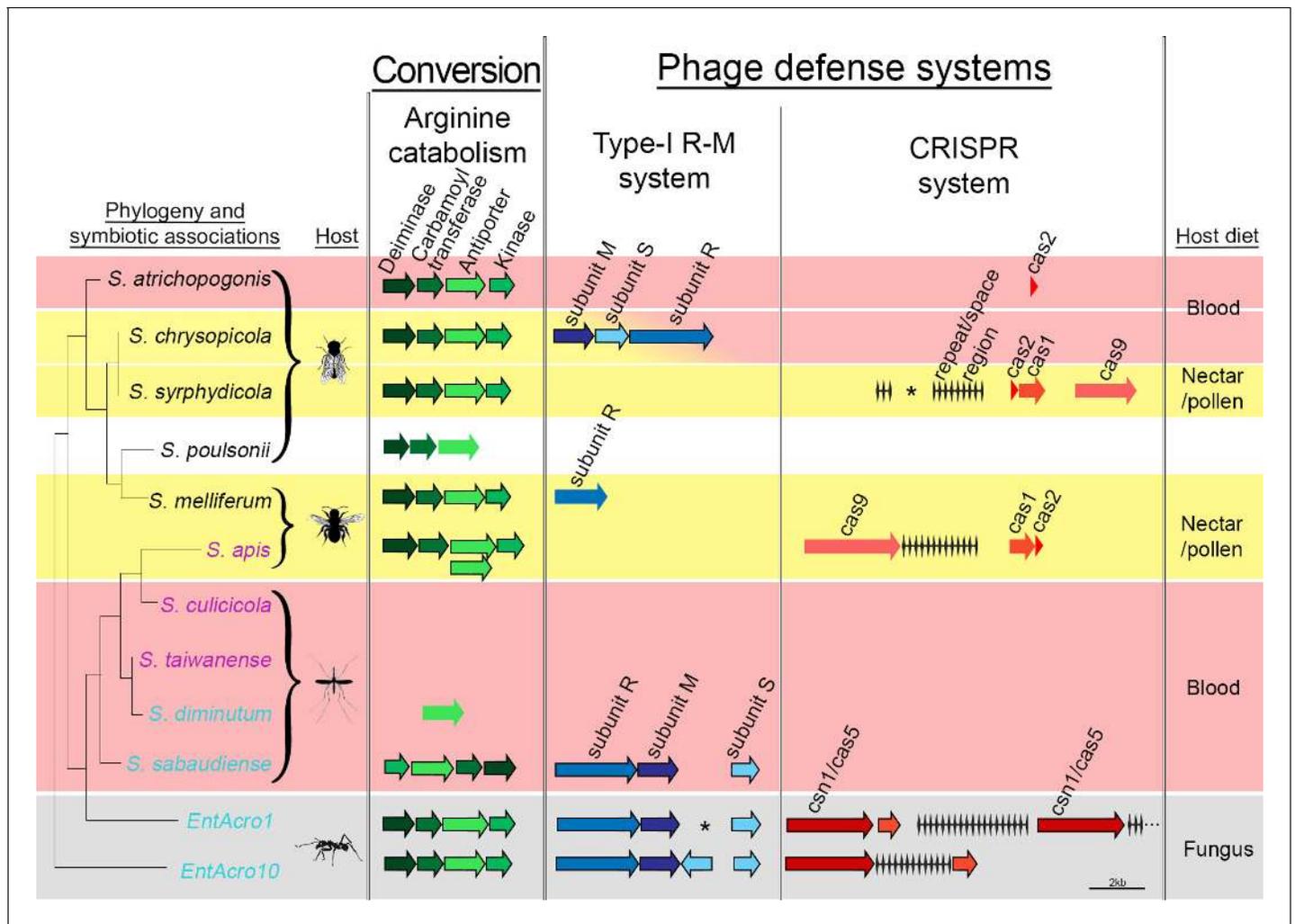


Figure 5. Organization of the arginine catabolism operon and two groups of bacteriophage-defense related genes (Type-I R-M and CRISPR systems) in *EntAcro1* and *EntAcro10* and closely related *Spiroplasma* symbionts of other insect hosts. The tree on the left and the color-coded species names specify different types of association (as in **Figure 1** with pink representing confirmed pathogenicity, light blue representing confirmed non-pathogenicity, and black indicating unknown but likely non-pathogenic; **Supplementary file 4**). Background colors highlight specialized host diets: fungus (gray), blood (red), nectar/pollen (yellow; mixed in deerflies hosting *S. chrysopicola* where males feed on nectar/pollen and females on blood) and unclear (white; food of *Drosophila* host is unknown but unlikely to be a specialized diet). Genes in each pathway have different shades of a single color and sizes of arrows and spaces between them are proportional to actual gene sizes relative to the 2 kb scale bar (bottom right). Gene orientations correspond to the direction of arrowheads and all genes are presented with their common names as identified by our RAST annotations (**Supplementary file 3**). Gene-arrows have a black outline when all core genes for a focal pathway are present, indicating it is functionally active. The asterisks for the Type-I R-M system in *EntAcro1* and the CRISPR system in the symbiont of *S. syrphydicola* indicate that the gap between the two arrows is not proportional to the scale bar but much wider.

DOI: <https://doi.org/10.7554/eLife.39209.020>

The following figure supplements are available for figure 5:

Figure supplement 1. Phylogeny of all amino acid transporters in *Saccharomyces*, *Agaricus bicolor*, *Coprinopsis cinerea*, *Schizophyllum commune* and the *Leucogarius* symbionts of *Atta*, *Acromyrmex* and *C. costatus*.

DOI: <https://doi.org/10.7554/eLife.39209.021>

Figure supplement 2. KEGG metabolic reconstructions based on the intact genes present in the *Acromyrmex*, *Solenopsis*, *Apis mellifera* and *Anopheles gambiae* genomes, together constituting the urea cycle.

DOI: <https://doi.org/10.7554/eLife.39209.022>

Figure supplement 3. Overview of the potential metabolic complementarity, inferred by our genomic reconstruction, between fungus-growing ants, their fungal cultivar, and their *EntAcro* symbionts.

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(Magni et al., 1999; Sánchez et al., 2008), suggesting that citrate catabolism by *EntAcro1* cells happens primarily in the midgut and in ileum where the pH is optimal for that function (Figure 4—figure supplement 1), which leaves arginine decomposition as the main terminal digestion process in the hindgut where pH is low.

Decomposition of all available hindgut arginine into NH_3 just before the anaerobic *EntAcro1* symbionts would die from exposure to aerobic conditions via the ants' fecal fluid would ensure that manure of the fungus-garden provides nitrogen in its most readily available form for fungal protein synthesis. The conversion of excess arginine to ammonia in the ant hindgut may thus resolve a potential mutualistic mismatch because the attine fungal cultivars have generic amino acid transporters, but they lack specialized arginine transporters to process environmental arginine, similar to other basidiomycete fungi (Figure 5—figure supplement 1). In general, ammonia is the preferred nitrogen source for fungal growth (Ahmad et al., 1990; Abril and Bucher, 2004), so any increase in the ammonia to arginine ratio of fecal fluid manure would benefit the farming symbiosis as a whole. At the same time this conversion prevents nitrogen waste, as would happen when excess arginine were to be deposited on a fungal cultivar primarily adapted to using simpler nitrogen sources.

Bacteriophage defense genes

We found clear evidence for both *EntAcro1* and *EntAcro10* having two intact bacterial defense systems to ward off phage attack, a Type-1 R-M system and a CRISPR pathway (Figure 5). Genes belonging to both defense systems are often horizontally transmitted among bacteria (Labrie et al., 2010) and maintaining them is costly (Stern et al., 2010; Vasu and Nagaraja, 2013; Vale et al., 2015; Burstein et al., 2016), so these defenses are primarily expected in bacterial species that face consistent threats of phage attack without being severely resource-constrained. Gene-level comparisons with the other *Spiroplasma* symbionts showed that none of them had the same dual defense system against phage attack. Type I R-M system genes were present only in non-pathogenic Mollicutes strains, similar to arginine catabolism pathways being restricted to *Spiroplasma* strains with non-pathogenic host associations, except for the reputedly pathogenic *S. apis* (Figure 5; Supplementary file 4). The generally rarer CRISPR system was complete (i.e. both CRISPR repeat/space regions and cas genes being present) only in *S. chrysopicola*, *S. apis*, and the two ant associated *EntAcro* strains.

Our finding that the two attine ant symbionts are unusually well protected is consistent with them being vulnerable to phage attack when they reach high densities in the gut lumen. We did indeed find *Spiroplasma*-specific phages of the Gokushovirinae in the contig bin 'C' of the *EntAcro1* symbiont (Supplementary file 1C) isolated from fecal fluid. Not finding these phage sequences in *EntAcro10* might reflect that these bacteria were isolated from the fat body of *A. dentigerum* where they are intracellular symbionts and that titers of *EntAcro10* were very low (Figure 3). These functional inferences are tentative, but potentially of significant interest, so we will return to them below.

Discussion

At the Panama site where we conducted our study, the *EntAcro1* and *EntAcro10* symbionts are the most common Entomoplasmatales strains associated with attine ants and they represent the majority of sequence reads (>40% jointly for both *EntAcro* symbionts that were obtained from these ants in field colonies and >50% in captive colonies fed *ad libitum*; Sapountzis et al., 2015). Our study thus captured much of the qualitative and quantitative biodiversity of abdominal Mollicutes endosymbionts. We show that these two symbionts are phylogenetically distant and therefore evolved independently (Figure 1), but that their gene contents reflect convergent adjustment to life as ant symbionts when compared to related *Mesoplasma* and *Spiroplasma* bacteria associated with other arthropods or plants (Figure 1—figure supplement 3). These convergences primarily relate to carbohydrate metabolism, consistent with patterns of bacterial adaptation being generally based on substrate utilization (Lo et al., 2015; Pál et al., 2005).

Several of the differences in metabolic genes between *EntAcro1* and *EntAcro10* relative to the *Mesoplasma* and *Spiroplasma* symbionts of the remaining insects emanate from rare genes that catabolize substrates such as citrate and arginine. These are complex molecules that attine ants and their fungal cultivars are likely to provide to their bacterial endosymbionts, but that are unlikely to be present in the food of other insects. None of the leaf-cutting ant colonies with a high prevalence

of Mollicutes symbionts ever showed any signs of pathogenicity, even when ants had several million Mollicutes cells in their bodies. Earlier studies left this issue open (Sapountzis et al., 2015; Meirelles et al., 2016), but the combined results of our present study clearly suggest that *EntAcro1* and *EntAcro10* are co-adapted mutualists and that their metabolic pathways shed novel light on several poorly understood aspects of the highly complex attine fungus-farming symbiosis.

The arginine recycling niche of Spiroplasma-like abdominal symbionts

The loss of the arginine synthesis pathway in the basal attine ants (Nygaard et al., 2011; Suen et al., 2011) has been instrumental in making their fungus-farming symbiosis obligate (Nygaard et al., 2016). The selection regime that caused this loss remains unknown (Nygaard et al., 2016; Ješovnik et al., 2016), but it is reasonable to assume that outsourcing the production of this most nitrogen-rich amino acid to fungal cultivars gave complementary efficiency benefits even though it also generated symbiotic dependency. For symbiotic division of labor to be sustainable under variable environmental conditions, average levels of fungal arginine production would have to be higher than the minimally sufficient level to avoid occasional windows of fatal shortage in the symbiosis as a whole. Symbiotic dependency may thus have created a niche for Mollicutes symbionts to ensure that surplus arginine is recycled as NH_3 to provide the most efficient manure for new garden growth.

Tissues of adult insects no longer grow and may thus only need small amounts of nitrogen for maintenance, so passing on excess NH_3 to fungus gardens via fecal fluid would have unambiguous benefits for the complex mutualism as a whole (Schjøtt et al., 2010). This conjecture was recently confirmed for *Atta* workers in an independent study showing that workers fed with ammonium nitrate (the protonated form of ammonia) transfer nitrogen via their fecal fluid to the fungus garden (Shik et al., 2018). Mollicutes-assisted garden manuring would thus imply that any surplus nitrogen remains a stable resource for new fungal protein synthesis and thus growth of the ant brood that only ingests fungal food. This underlines that driving-agency in obligate farming mutualisms is ambiguous. A shorter explanation of the prudence of this co-adaptation is that garden fungi domesticated ants to maintain and disperse them, and that they benefitted from the ants domesticating Mollicutes to ensure not a single nitrogen atom is wasted and their keepers could (later) utilize external resources such as citrate to work harder to enhance fungal growth.

The only other ant lineage in which Entomoplasmatales (Mollicutes) endosymbionts have so far been abundantly found are the army ants (Funaro et al., 2011). These ants are exclusive predators of mostly invertebrate prey (Kronauer, 2009) and 16S rDNA sequences of their Mollicutes symbionts suggested they are closely related to *EntAcro1* but rather distantly to *EntAcro10* (Funaro et al., 2011). It is intriguing that the Dacetine sister lineage of the fungus-farming ants are also specialized predators (Branstetter et al., 2017; Ward et al., 2015). It would thus be interesting to clarify whether also Dacetine ants have Entomoplasmatales symbionts, how (un)related they would be to the *EntAcro* symbionts of the fungus-growing ants, and whether army ants acquired their Mollicutes horizontally from preying upon on attine ants (Powell and Clark, 2004).

Broader consideration of the general feeding ecology of all insects hosting Mollicutes symbionts revealed that they are mostly specialized on nutritionally deficient but protein-rich diets, such as vertebrate blood (flies and mosquitoes) and pollen/nectar (bees and flies). The pollen/nectar feeders all had *Spiroplasma* symbionts with complete arginine catabolism pathways, suggesting they might similarly convert excess dietary arginine into NH_3 , although the absence of functional studies precludes speculation about the type of mutualistic advantage yielded by this conversion (de Groot, 1952; Vrzal et al., 2010; Uchida, 1993; Honeybee Genome Sequencing Consortium, 2006; Nene et al., 2007; Figure 5—figure supplement 2). Thus, while excreted NH_3 would appear to have a clear mutualistic function for manuring attine fungus-gardens (Figure 5—figure supplement 3), the benefits of NH_3 production in the gut system of bees and some mosquitoes is less clear. Overall, it seems that primarily non-pathogenic *Spiroplasma* strains may have been selected to catabolize host-food-associated arginine (Figure 5; Supplementary file 3; Supplementary file 4), but this provisional inference needs explicit functional verification by artificial diet experiments and selective removal of symbionts to quantify putative changes in arginine and ammonia titers.

Did the acquisition of *EntAcro1* facilitate the emergence of large-scale herbivory?

Our results indicate that *EntAcro1* was acquired as additional symbiont to *EntAcro10* relatively shortly before the leaf-cutting ants evolved and that *EntAcro1* supplements already available arginine recycling with novel pathways allowing ant workers to process non-fungal metabolites (**Figure 2**; **Figure 4**; **Figure 5—figure supplement 3**). Because citrate catabolism genes do not exist in *EntAcro10* the lower attine ants may be generally unable to convert plant-derived citrate or glucose/citrate into acetate. The acquisition of *EntAcro1* thus likely allowed the ant farmers to tap into additional non-fungal resources to maintain higher metabolic rates. These differences match what is generally known about the increases in farming scale, foraging activity, and garden growth-rates when moving from the basal attine ants to the derived branches of the attine phylogenetic tree (**Kooij et al., 2015**; **Shik et al., 2014**; **Shik et al., 2016**). It is interesting that one of the closest relatives of *EntAcro1* (*M. florum*) is associated with plants (**Figure 1**), which could suggest that this symbiont was acquired when the ancestors of the leaf-cutting ants started to forage on live plant material. However, confirmation of this hypothesis would require the closest relatives of *EntAcro1* to be associated with American Angiosperms. The few current records are from citrus trees of Asian origin (**Liu et al., 2012**), so substantial sampling effort will be needed to investigate this possible plant association.

The timing of the acquisition of *EntAcro1* is intriguing. It was recently shown (**Branstetter et al., 2017**) that a monophyletic crown group of the attine ants evolved in Central/North America following colonization of this subcontinent by a single South-American attine ancestor 22–27 MYA, well before the isthmus of Panama closed. The timing of isthmus closure is controversial with some maintaining that it happened as recently as ca. 3 MYA (**O’Dea et al., 2016**), while other studies indicate it may have been as early as the mid-Miocene ca. 13–15 MYA (**Bacon et al., 2015**; **Montes et al., 2015**). A recent study on army ants, whose queens are wingless and thus dependent on solid land-bridges for dispersal, indicated colonization of Central-North America 4–7 MYA (**Winston et al., 2017**). Also this dating is much later than the inferred timing of the first attine ant arrival in what was then the Central-North American subcontinent (**Branstetter et al., 2017**).

The implication is that most of the higher attine ant radiation happened on a novel subcontinent that was devoid of attine ants. In this context it is interesting that we also found *EntAcro1* in some field colonies of *T. cornetzi* (**Figure 3**), a higher non-leaf-cutting ant representing the most basal attine branch that colonized Central-North America (**Branstetter et al., 2017**). This suggests that *EntAcro1* may have been domesticated in Central-North America in response to the founding lineage encountering new ecological opportunities perhaps including plants with *EntAcro1*-like symbionts. Also here, larger sampling efforts will be needed to verify whether endosymbiotic microbiome signatures of this major biogeographic vicariance event continue to be found across the extant attine ants of Central/North and South America. If *EntAcro1* was domesticated just before or during the transition to active herbivory it may have somehow facilitated the transition to industrial-scale farming as presently found in the *Atta* and *Acromyrmex* leaf-cutting ants throughout the Americas.

The costs and benefits of defending domesticated bacterial symbionts in the gut

We found that both *EntAcro1* and *EntAcro10* have dual, fully intact cellular defenses against phage attack, consisting of a R-M (Restriction Modification) Type one system and a CRISPR pathway (**Figure 5**). We obtained a substantial number of phage sequences specific for *Spiroplasma*-like bacteria (the *EntAcro1C* bin; **Supplementary file 1C**) and our data show that the abundances of particularly *EntAcro1* inside ant bodies can be very high (**Zhukova et al., 2017**) (**Figure 3**). The mere presence of phage defensive mechanisms is not surprising because high clonal bacterial densities make bacteriophage attack efficient and rewarding. However, what makes the intactness of two complete pathways of the two *EntAcro* symbionts interesting is that none of the related *Spiroplasmas* associated with other insects has two such intact pathways. Both types of phage defenses are last resort systems, operating only when a phage has already broken through the bacterial cell membrane and has released its DNA into the bacterial cell. Recent work has clarified that these bacterial defenses are analogous to a non-specific innate immune system (R-M) and an adaptive (trainable) immune system

for recognizing specific viral DNA (CRISPR) (Vasu and Nagaraja, 2013; Seed, 2015), and that the two systems can operate synergistically (Dupuis et al., 2013). While ca. 90% of all bacterial genomes have at least one Restriction Modification system (Vasu and Nagaraja, 2013), less than 44% of bacterial genomes appear to have a CRISPR system (Burstein et al., 2016; Makarova et al., 2011) and these specific defenses are typically absent in obligate symbionts (Burstein et al., 2016).

It is increasingly documented that both types of phage-defense systems are likely to have fitness costs (Stern et al., 2010; Vasu and Nagaraja, 2013; Vale et al., 2015; Burstein et al., 2016). These costs may be expressed as slower growth in the absence of phages, somewhat analogous to the costs of autoimmune errors (Stern et al., 2010) and would be consistent with many bacterial lineages losing CRISPR genes relatively easily (Burstein et al., 2016). Lack of exposure explains why intracellular symbionts rarely have phage defenses compared to gut-lumen symbionts with much higher exposure to phages, which would also explain the presence of these systems in *EntAcro1*, an abundant gut lumen symbiont (Sapountzis et al., 2015). However the presence of these systems may also depend on a general trade-off between maintenance and growth. Preservation by active defense is much more likely to be a naturally selected priority for a vertically transmitted mutualist than for a pathogen selected to infect other colonies at the highest possible rate. Although tissue localization and proximate mechanisms such as the need to maintain chromosomal stability and recombination are important in determining the likelihood of acquisition and loss of phage-defense genes (Vasu and Nagaraja, 2013), the ultimate evolutionary cost-benefit argument is compelling enough to be spelled out for explicit testing in the future.

The endosymbiont-host interactions that we document include several feedback loops that should allow the ants to regulate *EntAcro* symbiont densities upwards or downwards depending on the overall costs and benefits of their services, similar to other hosts such as aphids which are able to control their intracellular *Buchnera* symbionts (Wilkinson et al., 2007; Russell et al., 2014). This underlines that phenotypic mechanisms for using symbiont services based on immediate cost-benefit ratios apply for both intra- and extra-cellular symbionts. Providing *EntAcro1* and *EntAcro10* symbionts with sufficient resources to maintain a full complement of phage-defense systems when they reach high densities would then appear to be a cost-efficient strategy to secure mutualistic services. This is because the only available route for propagation to future generations of a Mollicutes strain is to help maximize the colony's production of dispersing virgin queens (Meirelles et al., 2016). This would be achieved by host-induced optimization of bacterial titers rather than by maximal rates of bacterial cell division, in contrast to commensal or pathogenic bacteria that remain under selection to primarily maximize their rates of horizontal transmission (Frank, 1996). Comparative experimental tests measuring the phage-attack-sensitivity of intracellular and extracellular symbionts with and without phage defenses could be a way to verify these expectations that are consistent with the results presented here and with general evolutionary theory on levels-of-selection, efficiency of transmission, and the expression of competitive symbiont traits (e.g. Frank, 2012).

Note added in proof

A study by Gupta et al. (2018) that came online while our article was in the final stage of proof-checking performed a phylogenomic analysis of 121 conserved protein sequences in 32 Mollicutes genomes, of which some overlapped with the Mollicutes compared in our analyses. The Gupta et al. study complements the results presented in our paper by: 1. Confirming that *EntAcro1* and *EntAcro10* have very different origins, 2. Confirming that *EntAcro10* is the most basal branch of the Entomoplasmataceae and Spiroplasmataceae consistent with our Figure 1, 3. Confirming that the closest relative of *EntAcro1* is associated with plants – in their study a *Mesoplasma lactucae* isolated from lettuce corroborating the suggestion that the ancestors of the leafcutter ants may have acquired *EntAcro1* from plants on which they foraged, 4. Showing that citrus-associated *Mesoplasma florum* belongs to a more derived lineage closer to *Mycoplasma* strains consistent with our Figure 1, and 5. Placing the *Mesoplasma lactucae* strain in a new genus *Edwardiiplasma* together with *EntAcro1*.

Materials and methods

Rearing and handling of ant colonies

Apterostigma dentigerum and *Acromyrmex echinator* colonies were collected in Gamboa, Panama and maintained in rearing rooms at 25 °C and 70% relative humidity during a 12:12 hr photoperiod at the Centre for Social Evolution, University of Copenhagen, Denmark. The *Acromyrmex* colony (Ae331) used in this study had been kept under laboratory conditions for 8 years (collected in 2007), while the *Apterostigma* field colonies were all collected in May 2015, brought to the lab and sampled within the first two weeks from their field collection date. Some aspects of the composition of attine ant microbiota associated with the intestinal system may change after they are reared in the lab for a number of years, but the *EntAcro1* and *EntAcro10* symbionts are little affected and remain the two dominant Mollicutes symbionts across the attine ants at our field site in Gamboa, Panama also after colonies are transferred to the lab (*Sapountzis et al., 2015; Zhukova et al., 2017*).

Bacterial isolation, genome amplification and DNA extraction

Colony Ae331 from which we isolated the *EntAcro1* symbiont had been screened previously by 16S-Miseq sequencing and targeted 16S-PCR reactions, which showed its workers had high titers of *EntAcro1* and no detectable traces of the possible alternative strains *EntAcro2* or *EntAcro10* (*Sapountzis et al., 2015*). To obtain a pure sample of *EntAcro10* bacteria we used the lower attine ant *Ap. dentigerum* and performed an initial survey on 20 freshly collected colonies by extracting DNA from whole workers and performing PCR with *EntAcro10* specific primers (*Sapountzis et al., 2015*). This showed that workers from one colony (RMMA150520-03) were carrying the *EntAcro10* strain without any other Mollicutes being detectable. Prior to the further isolation of the two symbiont strains, a series of *Acromyrmex* and *Apterostigma* workers from these two colonies were anesthetized and surface sterilized by submergence in 70% ethanol for 1 min, after which they were rinsed twice in autoclaved MilliQ water, submerged in 50% bleach for 2 min, and rinsed again twice in autoclaved MilliQ water.

For the bacterial isolations we used a previously described protocol with some modifications (*Ellegaard et al., 2013; Iturbe-Ormaetxe et al., 2011*). For *EntAcro1* we obtained ca. 50 fecal droplets from *Ac. echinator* workers under a laminar flow hood (*Kooij et al., 2014b*) and deposited them in sterile petri dishes using sterile forceps, after which they were jointly suspended in 1000 µL cold SPG Buffer (218 mM sucrose, 3.8 mM KH₂PO₄, 7.2 mM K₂HPO₄, 4.9 mM l-glutamate, pH 7.2) and transferred to 1.5 ml Eppendorf tubes. To isolate *EntAcro10*, we dissected fat body cells from ca. 25 surface sterilized *Ap. dentigerum* workers under a stereomicroscope and immediately transferred them to a sterile 15 mL glass homogenizer (Wheaton) on ice, along with 1000 µL of cold SPG buffer. Using a glass pestle, we disrupted the tissues on ice and immediately transferred them into a new 1.5 ml Eppendorf tube.

The samples from both ant species were centrifuged at 4 °C for 15 min at 3,200 g after which the supernatant was transferred to new 1.5 ml microcentrifuge tubes and centrifuged again with the same settings. The supernatant was subsequently purified through a 5 µm (Acrodisc) and a 2.7 µm (Whatman) syringe filter, and finally through a 1.3 µm (Acrodisc) filter before transfer to a new 1.5 ml tube followed by centrifugation for 20 min at 18,000 g at 4 °C. The supernatant was discarded and the pellets (bacterial cells) were re-eluted in 5 µl SPG buffer. Approximately, 1 µl was then used for Multiple Displacement Amplification (MDA) to obtain whole genomic DNA using the Qiagen REPLI-g Midi Kit following the manufacturer's instructions. A blank reaction using sterile water as template instead of 1 µl of bacterial cell suspension was included in the same protocol to check for bacterial contaminations with eubacterial 515F/806R primers after the entire procedure was completed, which showed no detectable 16S amplicons.

The purified bacterial pellets used for MDA and subsequent dilution of the amplified DNA were subjected to PCR using the 16S generic primers 515F and 806R (*Caporaso et al., 2012*) as previously described (*Bourtzis and Miller, 2006*), purified using the Invitex kit (Westberg, Germany), and sent to MWG (Germany) for Sanger sequencing. After we had confirmed that the 16S amplicons were of Mollicutes' origin and that the chromatographs showed no signs of other bacterial 16S rDNA sequences, DNA was further purified using the Qiagen mini spin kit following the manufacturer's instructions. The extracted DNA was then quantified for both the *Ac. echinator* and *Ap.*

dentigerum sample using a Nanodrop spectrophotometer and sent to seqIT (Germany) where libraries were generated from 100 to 200 ng of DNA using the Nextera XT kit (Illumina, USA). Finally, MiSeq sequencing was performed at 2 times 250 bp read length, which generated approximately 3,000,000 reads per sample.

Assembly, annotation and quality controls

The Nextera adaptors used for the library construction were removed from the fastq files using Trim Galore (Babraham Institute) and the filtered reads were checked with FastQC (Andrews, 2016). We then used the SPAdes Genome Assembler (version 3.5.0) to generate a de novo assembly using the ‘-careful’ option which reduced the number of mismatches and short indels before running MismatchCorrector with kmer sizes of 21, 33, 55 and 77 to obtain a consensus assembly based on four individual assemblies (Bankevich et al., 2012). We then used the Burrows-Wheeler Aligner (BWA) to map reads to the assembled contigs (Li and Durbin, 2009), which produced a SAM file that was further analyzed using SAMTOOLS and converted to a BAM file that could be analyzed with Bamviewer v1.2.11 (Carver et al., 2010). The assembled contigs were further checked for errors using the Reap v1.0.18 software (Hunt et al., 2013) and contigs that had less than 9x coverage or were smaller than 250 bp were removed. The final set of assembled contigs (Supplementary file 1) was deposited in the NCBI Genome submission portal under accession numbers SAMN06251630 and SAMN06251631.

Genes for each contig were predicted using the RAST annotation server (Aziz et al., 2008) after which predicted amino acid sequences were compared to a local database Uniref100 using BLASTP v2.2.28+ (evalue <1e-15, percentage identities > 30%), and top matches with the assembled contigs were phylogenetically binned (Supplementary file 1). This grouped the contigs belonging to Mollicutes together in what we refer to as ‘A’ bins allowing further evaluation of the strain-specific RAST annotations (Simão et al., 2015). We functionally annotated the protein sequences using a stand-alone version of InterproScan v-5.17–56.0 with SUPERFAMILY, Pfam, ProSiteProfiles, Coils, ProSitePatterns, TIGRFAM, Hamap and ProDom (Jones et al., 2014) and Phobius (Käll et al., 2007) (<http://www.cbs.dtu.dk/services/SignalP/> and <http://phobius.sbc.su.se/>) to predict signal peptide and trans-membrane domains. To identify and compare metabolic pathways we used the KAAS tool (Moriya et al., 2007) provided by the KEGG database (Kanehisa and Goto, 2000; Kanehisa et al., 2010) together with the BLAST algorithm and the single best hit (SBH) procedure with default settings.

Phylogenomic analyses

For the phylogenomic reconstructions, we first downloaded all 176 available Mollicutes genomes that were present in the Ensembl database (page accessed in January 2016; (Yates, 2016)) and created a merged customized BLAST database, which we used to compare the predicted amino acid proteins of *EntAcro1A* and *EntAcro10A*. We included only complete genomes and thus excluded the partially sequenced *Entomoplasma melaleucaae* genome. The BLAST comparisons (Altschul et al., 1990) using an e-value of 1e-15 as cutoff and a percentage identity of 30%, revealed clear similarities between our two *EntAcro* symbionts and 59 previously genome-sequenced Mollicutes strains. We therefore used their genome sequences to define the orthologous single-copy protein-coding genes using the Orthofinder software (Emms and Kelly, 2015) which resulted in 73 genes being available for phylogenomic analyses. Both the nucleotide and amino acid sequences of these genes were extracted for each of the two *EntAcro* symbionts, which allowed the construction of gene-specific alignments using MUSCLE v3.8.31 (Edgar, 2004). These alignments were subsequently refined using the trimAl software, which removed all positions with gaps in 10% or more of the sequences unless this left fewer than 50% of the original sequences (Capella-Gutiérrez et al., 2009). The filtered alignments were further tested for recombination using the RDP software (Bruen et al., 2006) and for nucleotide saturation using the Xie test implemented in DAMBE5 (Xia and Xie, 2001). For the 65 genes that remained (Supplementary file 2) individual alignments were concatenated using Amas 0.98 (Borowiec, 2016) and the appropriate substitution models for the nucleotide and protein alignments were selected after testing them with jmodeltest v2.1.7 and protest v3.4 (Darriba et al., 2011).

We used the nucleotide and amino acid alignments for the two *EntAcro* symbionts and the other 59 Mollicutes genomes to reconstruct phylogenomic trees with maximum likelihood (ML) and Bayesian methods. For the ML analyses we used the RaxML software v.8.2.10 (Stamatakis et al., 2008) with specified partitions of the concatenated alignments, which produced 65 partitions (one for each gene) using the GTR model with invariable sites for the nucleotide alignment and the LG + IG model for the amino acid alignments (Le and Gascuel, 2008) after 1000 bootstrap sampling replications. For the Bayesian inferences, we used the MrBayes v3.2.6 software (Ronquist et al., 2012) applying the same nucleotide and amino acid models as above. The concatenated alignments were specified for each gene-specific partition and a variable rate of sequence evolution (ratepr) was allowed for each of them. Initially, five chains were run for one million generations and statistical samples were taken every 1000 generations, after which the analyses were repeated to cover a total of 10 million generations, because analysis of the effective sample sizes showed under-sampling in the initial trials. This produced an appropriate deviation of split frequencies (a standard measure in mrbayes which allows examination of how similar the calculated trees of two independent runs were) for the nucleotide (0.0014) and protein alignments (0.00001), with values well below the 0.01 threshold recommended as evidence for sufficient convergence and effective sample sizes exceeding 100 in all cases. All trees were further processed using FigTree v1.4.2 implemented in Geneious R7.1.1 (Kearse et al., 2012). The clipart images used in **Figure 1** were either obtained from wikimedia commons (<https://commons.wikimedia.org/>), or phylopic (<http://phylopic.org/>), available under a Public Domain License or drawn in Adobe Photoshop CS6.

Comparative genomics

We used the bactNOG database v4.5 (page accessed April 2016) to find clusters of orthologous genes and to compare the predicted proteins with HMMER v3.1.b1 (Eddy, 2011; Huerta-Cepas et al., 2016). To obtain specific comparisons among Mollicutes genomes based on different numbers of genes assigned to distinct functional categories via bactNOG, we obtained ratio estimates for the number of genes in each functional category by counting the number of genes assigned to specific orthogroups and dividing by the total number of annotated genes (proportional abundances), and used these data as input for Principal Component Analysis (PCA) using the 'stats' package in RStudio (v. 1.0.136).

We used Mantel tests to compare phylogenomic distances based on orthologous genes for insect hosts or bacterial symbionts with the overall genome dissimilarities. We focused on the eleven Mollicutes genomes (and their insect hosts) that we used for most of our genomic comparisons, because they were closely related. These were *EntAcro1*, *EntAcro10*, *S. sabaudiense*, *S. diminutum*, *S. culicicola*, *S. taiwanense*, *S. apis*, *S. melliferum*, *S. atrichopogonis*, *S. chrysopicola* and *S. syrphidicola*. We originally also included the *S. poulsonii* genome but the extremely high number of transposases (identified as ENOG410907Q, ENOG4105YCW, ENOG4105Y09, ENOG4105DQ6; Paredes et al., 2015) made this genome a clear outlier (also in the PCA ordinations presented in **Figure 1—figure supplement 3**) so we excluded it from further comparative analyses. We thus set out to compare 11 *Spiroplasma* and *EntAcro* strains with respect to: 1) their gene functional categories, 2) their bacterial phylogeny, and 3) their hosts' phylogeny. To compare gene functional annotations of the Mollicutes genomes, we used their orthogroup proportional abundances (see above) and created a dissimilarity matrix using Euclidean distance in R. For the bacterial phylogeny matrix, we used the phylogenomic distances constructed for this study based on the amino acid sequences of 65 orthologs (see above and **Figure 1**). For the genome-based host phylogeny matrix, we used a recent publication that constructed a global phylogeny for the holometabolous insects (Song et al., 2016). Whenever possible we used the same host species that the Mollicutes bacteria are associated with (*Apis mellifera*, *Aedes albopictus*, *Culex pipiens*), but when that was not possible we used the closest representatives within the same taxonomic clade for which a sequenced genome was available: *Simosyrphus grandicornis* instead of *Eristalis arbustorum*, *Cydistomyia duplonata* instead of *Chrysops. sp.*, *Culicoides arakawae* instead of *Atrichopogon*, and *Solenopsis geminata* instead of *Ac. echinator* and *Ap.dentigerum* (Ward et al., 2015). A host phylogeny for our matrix comparisons was then reconstructed using the selected mitochondrial genome sequences retrieved from NCBI (Accession numbers available in Supplementary Material of (Song et al., 2016) using MUSCLE v3.8.31 (Edgar, 2004) and FastTree v1.0 (Price et al., 2009). The final Mantel tests were performed in R using 10,000 permutations, which produced correlations between: 1. the functional annotation

matrix and the bacterial phylogeny matrix, 2. the functional annotation matrix and the host phylogeny matrix, and 3. the host and the bacterial phylogeny matrices.

Artificial diet experiments and reverse transcription quantitative PCR

To verify the putative mutualistic functions of the *EntAcro1* symbiont, we pursued two experimental approaches. First, we experimentally reduced the abundance of the symbionts in the bodies of ant workers and measured whether this had a negative effect on the acetate uptake activity of ant cells. We achieved this reduction by keeping ants on artificial diets that we knew from pilot experiments were either marginal or outright discouraging for the maintenance of Mollicutes endosymbionts. For these experiments we used four different colonies of *A. echinator*: Ae150, Ae331, Ae360 and Ae507, which had been kept in the lab for 15, 9, 8 and 5 years respectively, before the experiment. Ant workers were removed from their fungus gardens and placed in sterile petri dishes with an inverted screw-lid in the middle filled with the nutrition-medium of choice. Using the known composition of mango fruit juice (*Medlicott and Thompson, 1985*), a resource that leaf-cutting ants at our field site regularly utilize, we created an artificial diet consisting of 140 mM sucrose, 130.6 mM fructose, 70 mM glucose and 4 mM sodium citrate (Sigma aldrich, Denmark). This diet was offered to the ants for seven days either in pure form or with 1 mg/ml tetracycline and 1 mg/ml rifampicin added. As controls we used workers picked directly from their fungus-garden.

We examined the expression of relevant genes in three type of tissues of ant workers: (1) approximately 50 fecal droplets from the rectum; (2) fat body, midgut and part of the ileum tissues of 20 ants; and (3) heads and thoraxes of 5 ants to represent the remaining body parts as controls. We excluded all samples originating from the heads and thoraxes from further analyses, because we never detected any bacterial gene expression in them except for a single sample (*ftsZ* gene expression in colony Ae360). We validated the effect of our diet manipulation by measuring the expression of *ftsZ*, a single copy bacterial gene that we amplified with qRT-PCR using *EntAcro1*-specific primers (*Supplementary file 5*). To evaluate acetate import activity by ant cells, we measured the expression of an MCT-1 ortholog that has been demonstrated to mediate acetate uptake in animals (*Kirat and Kato, 2006; Moschen et al., 2012*) as well as nine other MCT-like genes in the *Ac. echinator* genome that are predicted to import multiple short-chain fatty acids (SCFAs) potentially including acetate (www.uniprot.org; *Supplementary file 6*). To measure the expression of MCT-like genes and the number of *EntAcro1* cells (by measuring expression of the bacterial housekeeping gene *ftsZ*), we only used fat body and midgut tissue samples (which also included part of the ileum), since we could not detect any expression of MCT-like genes in the rectum lumen (not surprising because the rectum lumen has no ant cells). To evaluate the statistical significance of the correlation between expression of each MCT or MCT-like gene and *ftsZ*, we used non-parametric Spearman correlation tests on the $\Delta\Delta C_T$ values.

In a second set of experiments using the same type of ant tissues as above, we compared the expression of four predicted *EntAcro1* transporter genes (arginine, GlcNAc, citrate, glycerol or DHA; see *Figure 4—figure supplement 1*) in the midgut and fat body tissues relative to the (hindgut) rectum lumen to obtain insight in the metabolic activity of *EntAcro1* symbionts throughout the alimentary tract where pH is known to change from ca. neutral to acid (ca. pH 4) conditions just before defecation (*Erthal et al., 2004*). For this experiment, we also sampled 50 ant workers from the same four *Ac. echinator* colonies (Ae150, Ae331, Ae360 and Ae507). To test the effect of the gene identity and tissue compartment on the expression data, we used an ANOVA linear model with the $\Delta\Delta C_T$ values (ratio of target gene expression/*ftsZ* gene expression) from the qPCR as response variable and the predicted transporter gene identity (arginine, citrate, GlcNAc and glycerol/DHA), the tissue identity (midgut/fat body or hindgut) and their interaction as fixed factors using the function 'aov' in R (*Chambers and Hastie, 1992*). We evaluated significant differences across groups (transporter gene and gut compartment) using post-hoc comparisons as planned contrasts and Bonferroni corrections based on the 'glht' function in the 'multcomp' package (*Hothorn et al., 2008*). Before performing these tests, we visually examined normality of the data distribution ('hist' command) and confirmed impressions of no significant deviations with Shapiro–Wilk tests, which gave no indications of heteroskedasticity or deviations from normality that would compromise the validity of parametric statistics.

Technical procedures in both experiments were as follows: All tissues were dissected and collected in ice-cold RNAlater and stored at -80°C until extraction after which total RNA was extracted

using an RNeasy minikit (Qiagen, Germany). Fifty-five ng of extracted RNA was treated with RQ1 RNase-free DNase I (Promega Corporation, Madison, WI), and 50 ng of the resulting product was reverse transcribed with an iScript RT kit (Bio-Rad Hercules, CA, USA) to obtain first-strand cDNA. As a negative control, the remainder of the DNase-treated RNA was examined by PCR under the same conditions. All gene-specific PCRs were performed on cDNA, DNase-treated RNA, ant DNA, and water as in previous procedures (Andersen *et al.*, 2012; Sapountzis *et al.*, 2015). For the qPCR reactions we used the SYBR Premix Ex Taq PCR mix (TaKaRa Bio Inc., St. Germain en Laye, France) on the Mx3000P system (Stratagene, Santa Clara, CA, USA). Reactions took place in a final volume of 20 μ l containing 10 μ l buffer, 8.3 μ l sterile double-distilled water (ddH₂O), 0.4 μ l of each primer (10 mM), 0.4 μ l ROX standard, and 0.5 μ l template cDNA. PCR conditions were as follows: denaturation for 2 min at 94°C, followed by 40 cycles of 30 s at 94°C, 30 s at the annealing temperature (see **Supplementary file 5** for primers used), and 30 s at 72°C, followed by dissociation curve analysis. All quantitative PCRs (qPCRs) were replicated and each run included two negative controls with no added template.

To generate the delta values for the qPCR analyses, we used the fold change method, based on a standard curve with PCR products in a tenfold dilution series of known concentrations to calculate the PCR efficiency of each primer pair using the REST software (Pfaffl, 2002). Data were then imported into R and expressed as $\Delta\Delta C_T$ values, that is, as fold changes relative to the *Ac. echinator* specific *rpl7* housekeeping gene for the ant host and the *ftsZ* gene specific for the *EntAcro1* symbiont (Pfaffl, 2001).

Estimation of absolute abundances of EntAcro1 and EntAcro10 in ant hosts and other statistical analyses

Pooled abdominal tissues from five ant workers were collected from five lab colonies of *At. colombica*, four colonies of *At. cephalotes*, four colonies of *At. sexdens*, five colonies of *Ac. echinator*, four colonies of *Ac. octospinosus*, five colonies of *T. cornetzi*, five colonies of *T. zeteki*, five colonies of *S. amabilis* and four colonies of *Ap. dentigerum*. These samples were supplemented with ca 5–15 whole worker gaster samples (abdomens minus the first segments that are integrated in the thorax or form the well-known hymenopteran constriction that has no organs) of attine ant species that were too small to dissect: five lab colonies of *C. costatus*, three colonies of *C. rimosus*, three colonies of *Myr. ednaella* and five colonies of *Myc. smithii*. All samples were stored in -20°C and DNA was extracted based on previously described methods (Sapountzis *et al.*, 2015).

We estimated the abundances of Mollicutes symbiont DNA using qPCR with primers targeting the 16S gene of *EntAcro1* (Sapountzis *et al.*, 2015) and *EntAcro10* (Supplementary file 5). For each 16S gene that we analyzed, the initial template concentration was calculated from a standard curve with PCR product in tenfold dilution series of known concentration, as quantified by nanodrop. Since our genomic data showed that one 16S copy corresponded to one *EntAcro1* or one *EntAcro10* cell, we first calculated the results as numbers of bacterial symbiont cells per ant. We chose not to normalize the data using a specific single copy ant gene (such as EF-1a used in other studies; Sapountzis *et al.*, 2015), because samples were from different tissues, that is, either dissected fat bodies and midguts for the ant species with larger body size or whole gasters for the small-bodied species. However, we did obtain data on species-specific fresh-weight body mass of workers and used them to approximately scale the bacterial cell counts. To estimate mean worker body mass per attine ant species, we weighed five random workers from three colonies from each species to obtain the following average values: *A. colombica* 4 mg, *A. cephalotes* 4.1 mg, *A. sexdens* 4.7 mg, *A. echinator* 9.7 mg, *A. octospinosus* 12.5 mg, *T. cornetzi* 1 mg, *T. zeteki* 1.9 mg, *S. amabilis* 1.2 mg, *C. costatus* 0.1 mg, *C. rimosus* 0.4 mg, *M. ednaella* 0.3 mg, *M. smithii* 0.2 mg and *A. dentigerum* 2.2 mg.

To compare the abundance levels of *EntAcro1* and *EntAcro10* per unit of worker body mass, we used a generalized (negative binomial) linear model (GLM) with the function 'glm.nb' in the package 'MASS' (Venables and Ripley, 2002). This model was a better fit than a GLM model with gamma or Poisson distribution when we compared models according to the Akaike Information Criterion (AIC). We used the absolute abundance values (bacterial counts normalized per unit of ant biomass) as response variable, and bacterial strain (i.e. *EntAcro1* or *EntAcro10*), phylogenetic host group (i.e. leaf-cutting or non-leaf-cutting ants), and the statistical interaction between these predictors as fixed categorical variables. We evaluated significant differences across groups using post-hoc comparisons

as planned contrasts and Bonferroni corrections based on the 'glht' function in the 'multcomp' package (Hothorn et al., 2008).

Collection of forage substrates harvested by colonies in the field

Foraging substrate preference data were collected in the field from nine attine ant species (*T. cornetzi*, *T. zeteki*, *S. amabilis*, *C. costatus*, *C. longiscapus*, *C. rimosus*, *Myc. smithii*, *Myr. ednaella* and *Ap. dentigerum*) in Gamboa, Panama and represent 101 hr of observation time on 103 colonies (*T. cornetzi* (n = 48), *T. zeteki* (n = 12), *S. amabilis* (n = 6), *C. costatus* (n = 8), *C. longiscapus* (n = 5), *C. rimosus* (n = 8), *Myc. Smithii* (n = 14) and *Ap. dentigerum* (n = 2). Colonies were located and marked in several field sites within lowland Panamanian rainforest near Gamboa after placing polenta baits in the leaf litter and then tracking workers back to their nests when vouchers of workers were collected in EtOH to allow identification. After at least a week, laden returning foragers were observed with a headlamp during set observation periods. Colonies were typically observed during 60 min intervals (59 ± 14 min), although observations were cut short in the case of rain, or extended in the case of very slow foraging (e.g. 120 min intervals for *Ap. dentigerum*). Harvested substrates were carefully removed from the mandibles of the workers, collected in eppendorf tubes, and returned to the lab where they were dried at 60°C for 24 h and then sorted under a dissecting microscope and catalogued. Substrates collected by the ants were split in seven categories (leaves, fruits, flowers, seeds, wood fragments, insect frass and 'other', that is, small dead insect fragments, pieces of unidentifiable detritus, and putative bird feces) for which counts per observed colony were generated. Similar data were extracted from a recently published study conducted at the same field site at the same time of year (May) but focusing on six leaf-cutting ant species (*At. colombica*, *At. cephalotes*, *At. sexdens*, *Ac. echinator*, *Ac. octospinosus* and *A. volcanus*) in Gamboa, Panama (Kooij et al., 2014a). Merged datasets were normalized by converting all observations to total observed counts in one hour.

To visualize foraging substrate preferences across the attine ant species, we converted the count data to proportions and performed an unscaled Principal-Component Analysis (PCA) in R using the 'ade4' package. To independently verify the statistical significances obtained, we used the mean of the replicated count values per colony. We then fitted the normalized hourly count data in a zero-inflated negative binomial (ZINB) regression model with the function 'zeroinfl' in the package 'pscl' (Kleiber et al., 2008). ZINB regression is typically used for true count variables to model positively-skewed data with an abundance of zeros and it fitted our data better than a zero-inflated Poisson or a negative binomial generalized linear model (GLM) without zero-inflation when we compared them using the Akaike Information Criterion (AIC) and the Vuong's non-nested test ('vuong' function in 'pscl' package). We used the absolute itemized foraging substrate preference values as response variable and the interaction of the foraging substrate types (i.e. leaves, fruits, flowers, seeds, insect frass and other) and phylogenetic group (i.e. leaf-cutting versus non-leaf-cutting ants) as fixed categorical variables. We conducted Tukey's HSD post hoc tests for each substrate type between leaf-cutting and non-leaf-cutting using the 'lsmeans' package (Lenth, 2016).

We used Mantel tests to compare differences in absolute *EntAcro1* or *EntAcro10* abundances (calculated for each species using the qPCR data) with the overall dissimilarities in their foraging substrate preferences. We used only data from the 12 attine ant species which were common in both datasets (*At. colombica*, *At. cephalotes*, *At. sexdens*, *Ac. echinator*, *Ac. octospinosus*, *T. cornetzi*, *T. zeteki*, *S. amabilis*, *C. costatus*, *C. rimosus*, *Myc. smithii* and *Ap. dentigerum*). We created a bray-curtis distance matrix for each of the *EntAcro* strains and a similar dissimilarity matrix based on the the seven foraging substrate categories using bray-curtis distance in R. The final Mantel tests were performed in R using 10,000 permutations.

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Author contributions

Panagiotis Sapountzis, Conceptualization, Data curation, Formal analysis, Funding acquisition, Validation, Investigation, Methodology, Writing—original draft, Writing—review and editing; Mariya Zhukova, Validation, Methodology; Jonathan Z Shik, Resources, Data curation; Morten Schiott, Supervision, Validation; Jacobus J Boomsma, Conceptualization, Resources, Supervision, Funding acquisition, Validation, Writing—original draft, Project administration, Writing—review and editing

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Additional files

Supplementary files

- Supplementary file 1. Summary statistics of the metagenomic sequencing of the endosymbiotic Mollicutes *EntAcro1* and *EntAcro10*. Following the assembly, contigs were separated into phylogenetic bins, which are presented in each column. **(A)** Sequencing statistics, annotation and completeness assessment using the BUSCO orthologs. **(B)** Total numbers of predicted coding sequences (CDS) that gave top matches with different bacterial classes for *EntAcro1*. **(C)** Comparable data for the *EntAcro10* bins. In both data sets, 'A' bins were the ones that contained contigs with clear similarities to Mollicutes, while 'B' bins mostly represented sparse contaminants, and the 'C' bin (only *EntAcro1*) contained sequences that belonged to bacteriophages of the family *Microviridae* (see text for details). Following the results of the BUSCO analysis, we examined the complete genome sequence of the *M. florum* symbiont associated with plants (**Baby et al., 2013**) using the same

software, which gave the same numbers of genes as for *EntAcro1*. These results suggested that our 'A' bins represent proper draft genomes of single *EntAcro1* and *EntAcro10* strains.

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- Supplementary file 2. The 65 single-copy orthologs used in our phylogenetic reconstructions as identified by Orthofinder based on 59 Mollicutes genomes, associated mainly with mammal, insect and crustacean hosts (see Materials and methods). Genome names are presented at the top of each column with the same identifiers as in the Ensembl database (<http://bacteria.ensembl.org/info/web-site/ftp/index.html>). NCBI accession numbers are given for all ortholog genes (rows) except for *EntAcro1* and *EntAcro10* where we used the RAST annotation identifiers.

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- Supplementary file 3. Differences in predicted coding sequences of the fungus-growing ant symbionts *EntAcro1A* and *EntAcro10A* and the genomes of closely related *Spiroplasma* strains associated with mosquito, honeybee and fly hosts that also have specialized diets. The main table (**A**) shows the predicted set of genes always present in *EntAcro1A* while at the same time overlapping with *EntAcro10A* (the 39 top rows) and to a variable extent also with the twelve other *Spiroplasma* strains (73 bottom rows). KEGG annotation, eggNOG functional and hierarchical categories, subcategories, subsystems and roles for the predicted genes, as defined by the RAST annotation server, are presented as columns while rows show presences and absences. *EntAcro1A* genes were sorted first for being shared or not with *EntAcro10A*, then with the mosquito-associated *S. sabaudiense*, *S. diminutum*, *S. culicicola*, *S. taiwanense*, then with the bee-associated *S. melliferum* and *S. apis*, and finally with the fly-associated *S. atrichopogonis*, *S. chrysopicola*, *S. syrphidicola*, and *S. poulsonii*. Presences of genes in strains documented or believed to be pathogenic are given in pink and presences in putatively mutualistic or commensal strains are given in light blue (same colors as in **Figures 1** and **5**). For strains without clear literature references indicating pathogenicity (implying they are unlikely to be pathogens; **Supplementary file 4**) we used a dark gray font. Enriched complete pathways of *EntAcro1* are highlighted with different background colors across rows, using gray and black for complete pathways that are common in bacteria and usually have housekeeping functions or are involved in homologous recombination and are thus not of primary interest. Complete pathways that were of focal interest (based on the eggNOG annotation) are marked in bright colors: shades of green for those involved in metabolism, red for those involved in information processing, and blue for those related to cellular function (same as in **Figure 1—figure supplement 1**). The legend directly below the table provides short descriptions for each of the highlighted pathways. The separate table (**B**) summarizes the gene-distributions for the pathways described in Table A, with regard to: (i) pathogenic and non-pathogenic associations of Mollicutes strains, (ii) the respective hosts strains they are associated with, and (iii) bacterial phylogeny (**Figure 1**).

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- Supplementary file 4. Symbiotic associations of the Mollicutes strains for which sequenced genomes were available for phylogenetic comparison with the focal *EntAcro1* and *EntAcro10* symbionts of the present study. From left to right: the 59 Mollicutes strain IDs for which a phylogeny was constructed (**Figure 1**, **Figure 1—figure supplement 2**) using the same identifiers as in the Ensembl database (<http://www.ensembl.org>), their most likely symbiotic associations (based on the available literature), their hosts, and previous studies that examined their symbiotic interactions.

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- Supplementary file 5. Primers used in the present study. From left to right: Primer names, the function of the gene targeted based on the RAST annotation, the target organism, the primer sequences from 5' to 3' and the annealing temperatures used for the qPCR.

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- Supplementary file 6. Correlation statistics for the association between the number of *EntAcro1* cells and the expression of 10 MCT-like genes in the *Ac. echinator* midgut and fat body tissues. From left to right we present for each gene examined: The protein ID in the Uniprot database (<http://www.uniprot.org/>), the gene name and the gene ID based on the *Ac. echinator* annotation, the predicted protein size, and the results of statistical analyses examining the correlation between expression of the bacterial *ftsZ* gene in midgut/fat body tissues and the expression of MCT-like genes. The significance of each correlation was first evaluated using a non-parametric Spearman correlation test (see Methods), for which we give ρ values, p values and Bonferroni corrected p values.

The only significant correlation (marked in bold) was between the *ftsZ* and the MCT1 (F4WHWZ) transporter, for which previous functional experiments have demonstrated that it is actively involved in the import of acetate in eukaryotic cells (*Kirat and Kato, 2006; Moschen et al., 2012*).

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• Transparent reporting form

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Data availability

Sequencing data were deposited in the NCBI Genome submission portal under accession numbers SAMN06251630 and SAMN06251631.

The following datasets were generated:

Author(s)	Year	Dataset title	Dataset URL	Database and Identifier
Panagiotis Sapountzis	2015	Entomoplasmatales bacterium EntAcro1	https://www.ncbi.nlm.nih.gov/biosample/?term=SAMN06251630	NCBI BioSample, SAMN06251630
Sapountzis P	2015	Entomoplasmatales bacterium EntAcro10	https://www.ncbi.nlm.nih.gov/biosample/?term=SAMN06251631	NCBI BioSample, SAMN06251631

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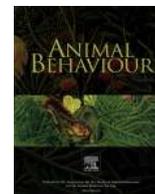
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The scent of symbiosis: gut bacteria may affect social interactions in leaf-cutting ants

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Animal gut microbiota affect host physiology and behaviour. In social insects, where colony level integrity is preserved via a nestmate discrimination system based on cuticular hydrocarbon mixtures, microorganismal effects may therefore influence social dynamics. Although nestmate recognition has undergone a thorough exploration during the last four decades, few studies have investigated the putative role of gut microbes. Here, we integrated 16S rRNA-based microbial community profiling, chemical and behavioural approaches to test whether gut microbes affect nestmate recognition in *Acromyrmex echinator* leaf-cutting ants. Treating workers with a sterile diet or with antibiotics resulted in a substantial alteration of their gut microbial communities. In pairwise social interactions, untreated versus antibiotic-treated nestmates behaved more aggressively than other nestmate and non-nestmate pairs, suggesting that bacterial suppression may alter chemical social cues and trigger aggressive behaviour. Chemical analyses of treated individuals revealed a decrease in the abundance of two metapleural gland antifungal compounds, and confirmed the correspondence between aggression levels and chemical profile differences. Feeding microbiota-remodelled ants with conspecific faecal droplets partially restored the original bacterial communities. Ants fed with faecal droplets from different colonies were unusually aggressive compared to pairs fed with faecal droplets from the same colony. We cannot exclude confounding effects resulting from the potentially harmful action of antibiotics on ant hosts. However, our results suggest a correlation between chemical profiles and the presence of certain microbial species in the gut, which may affect nestmate recognition and division of labour. This opens novel questions about the role of symbiotic microorganisms in the evolution of social insect behaviour.

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In the evolution of mutualistic relationships between meta-organisms and prokaryotes, animals have often co-opted the metabolic versatility of microbes to upgrade their physiology, while

microorganisms have found favourable environments in animal bodies. As part of the physiology of their animal hosts, symbiotic microbes are also involved in their behavioural processes (Archie & Theis, 2011; Archie & Tung, 2015; Cryan & Dinan, 2012; Dance, 2014; Ezenwa, Gerardo, Inouye, Medina, & Xavier, 2012). Many insightful discoveries about the physiological and behavioural effects of symbiotic microorganisms stem from the study of germ-free or germ-remodelled animals. Research comparing germ-free mice to their untreated counterparts has revealed that microbial gut symbionts affect anxiety-like behaviour (Clarke et al., 2013; Heijtz et al., 2011; Neufeld, Kang, Bienenstock, & Foster, 2011) and social interactions (Buffington et al., 2016). Similarly, an increasing corpus of *Drosophila* studies suggests that gut microbes

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mediate a plethora of physiological/behavioural processes, including specific appetites for proteins (Leitão-Gonçalves et al., 2017), mate choice and mating dynamics (Arbuthnott, Levin, & Promislow, 2016; Morimoto, Simpson, & Ponton, 2017; Ringo, Sharon, & Segal, 2011; Sharon et al., 2010) and the recognition of kin and familiar individuals (Lizé, McKay, & Lewis, 2014).

The increasing awareness of the behavioural role of microbes begs questions about how microorganisms may affect the behavioural ecology of animals with advanced superorganismal colony life. Such insects, the ants, corbiculate bees, vespine wasps and higher termites, live in family groups with permanent division of reproductive labour, unmated worker castes and sophisticated chemical communication (Gadau & Fewell, 2009). At the same time, the collective resource acquisition behaviours of the workers are reminiscent of differentiated somatic cells building a metazoan body, but now elevated to the level of organized collective foraging, brood nursing, nest construction and colony defence (Boomsma & Gawne, 2018; Wheeler, 1928). Across taxa, much of the maintenance of collective social integrity and sustainable infrastructure is ensured by chemically mediated nestmate recognition systems, which rely primarily on cues emanating from long-chain hydrocarbons embedded in the waxy layer of the insect cuticle (henceforth cuticular hydrocarbons, or CHC).

Scattered studies have provided evidence for bacterial ecto- or endosymbionts (i.e. symbionts living outside or inside the body of the host of the host, either intra- or extracellularly; Bourtzis & Miller, 2003) potentially affecting CHC-mediated behaviours, but without producing a coherent consensus. *Reticulitermes speratus* fed with bacteria extracted from termites of unrelated colonies were attacked by their nestmates, and manipulation of bacterial communities with antibiotics also affected nestmate recognition behaviour (Matsuura, 2001). In contrast, workers of *Pogonomyrmex barbatus* harvester ants with experimentally augmented cuticular microbiomes were more often rejected by nestmates, but workers smeared with antibiotics were not discriminated against. This suggested that only cuticle-dwelling microbes might influence nestmate recognition dynamics (Dosmann, Bahet, & Gordon, 2016). However, smearing the cuticles of workers of the leaf-cutting ant *Acromyrmex subterranean subterranean* with antibiotics did not affect cuticular hydrocarbon profiles (de Souza et al., 2013). Similarly, a study on *Camponotus fellah* carpenter ants showed that workers with high titres of *Blochmannia* intracellular endosymbionts had rather low overall quantities of CHCs but with the same relative proportions of the constituent compounds (de Souza, Devers, & Lenoir, 2011). Finally, feeding workers of the Argentine ant *Linepithema humile* with antibiotics affected their interspecific social interactions but not the behaviours expressed towards workers of other colonies of their own species, suggesting that bacterial endosymbionts do not significantly affect nestmate recognition in this ant species (Lester, Sébastien, Suarez, Barbieri, & Gruber, 2017).

Despite providing insights into the possible roles of bacterial symbionts in CHC-mediated nestmate recognition, previous studies either investigated microbial effects with behavioural tests but without measuring changes in CHC profiles (Lester et al., 2017; Matsuura, 2001), or they compared CHC profiles between antibiotic-treated and control workers without monitoring the correlated social behaviours (de Souza et al., 2011, 2013; Dosmann et al., 2016). To obtain more encompassing insights into the interplay between gut microbiota and chemically mediated social interactions, we performed an integrated analysis of all three variables: CHC profiles, gut bacterial communities and nestmate recognition behaviour. We experimentally changed the previously characterized gut microbiota of *Acromyrmex echinator* leaf-cutting ants (Sapountzis et al., 2015; Sapountzis, Zhukova, et al. 2018; Van Borm, Billen, & Boomsma, 2002) to investigate correlated changes

in socially relevant chemical cues and aggressive behaviour. In a first set of experiments (Round 1), we either mildly or strongly suppressed the native gut microbiota of *A. echinator* workers using, respectively, a sterile sucrose diet or an antibiotics diet. We then measured the changes in the abdominal microbial communities of worker ants and their cuticular chemical profiles and used dyadic aggression trials to evaluate associated changes in aggressive behaviour. In a second set of experiments (Round 2), we partially restored the original abdominal microbial communities of worker ants and measured the extent to which changes in CHC-profiles and aggression could be reversed. To achieve these effects, we fed experimentally manipulated worker ants with faecal droplets of untreated conspecifics and analysed their abdominal microbial communities, chemical profiles and aggression behaviours.

METHODS

Ethical Note

As no ethical restrictions apply to insect research under European legislation, no institutional or governmental regulatory bodies reviewed this study.

Experimental Design

We used workers from four *A. echinator* colonies (Ae150, Ae322, Ae153 and Ae331, hereafter named A, B, C and D, respectively) with an already characterized abdominal microbial community (Andersen, Hansen, Sapountzis, Sørensen, & Boomsma, 2013; Sapountzis et al., 2015; Sapountzis, Zhukova, Shik, Schiøtt, & Boomsma, 2018) for the experiments (Fig. 1). Colonies were collected in Gamboa, Republic of Panama in 2003–2010, and kept at ca. 25 °C, 70% relative humidity and 12:12 h light:dark photoperiod. Individuals were taken from fungus gardens (375 individuals/colony, $N = 1500$) and placed in groups of 15 in sterile petri dishes (diameter 90 × 15 mm; 25 dishes/colony, $N = 100$) including a liquid food vial. For each colony, we randomly assigned workers to two treatment groups: (1) ants kept on a sterile 10% sucrose solution diet (150 individuals/colony); and (2) ants kept on the same sucrose solution supplemented with 1 mg/ml of the antibiotic tetracycline (225 individuals/colony). After 2 weeks, 522 workers underwent a series of tests (Round 1) including aggression assays and analyses of CHCs (GC–MS, see below) and gut microbiota (qPCR and MiSeq, see below). The remaining workers were used in a second series of tests (Round 2), where we attempted to restore their original microbiota by feeding them with a sterile 10% sucrose solution supplemented with faecal droplets (0.033 droplets/μl) obtained by squeezing abdomens of untreated workers from the source colonies. Colony B and D workers received faecal fluid from nestmate workers, whereas colony A and C workers received faecal fluid from non-nestmate workers (i.e. from colonies B and D, respectively, Fig. 1). After 1 week, individuals were tested in aggression assays and used for CHC and microbiota analyses. Throughout the experiments, we monitored survival to determine the effects of our treatments on ant mortality (Appendix Fig. A1).

Finally, we set up an independent smaller experiment to compare the microbial communities of guts, heads and thoraxes of a subset of workers from all treatment groups, before and after the experimental diet treatments. This experiment included workers from three out of four ant colonies used in our main experiment (A, B and D), in an identical set-up. The only difference was that we used not only guts but also heads and thoraxes for bacterial characterization. This aimed to make sure we had not overlooked significant microbial presences in other body parts (Appendix Fig. A2).

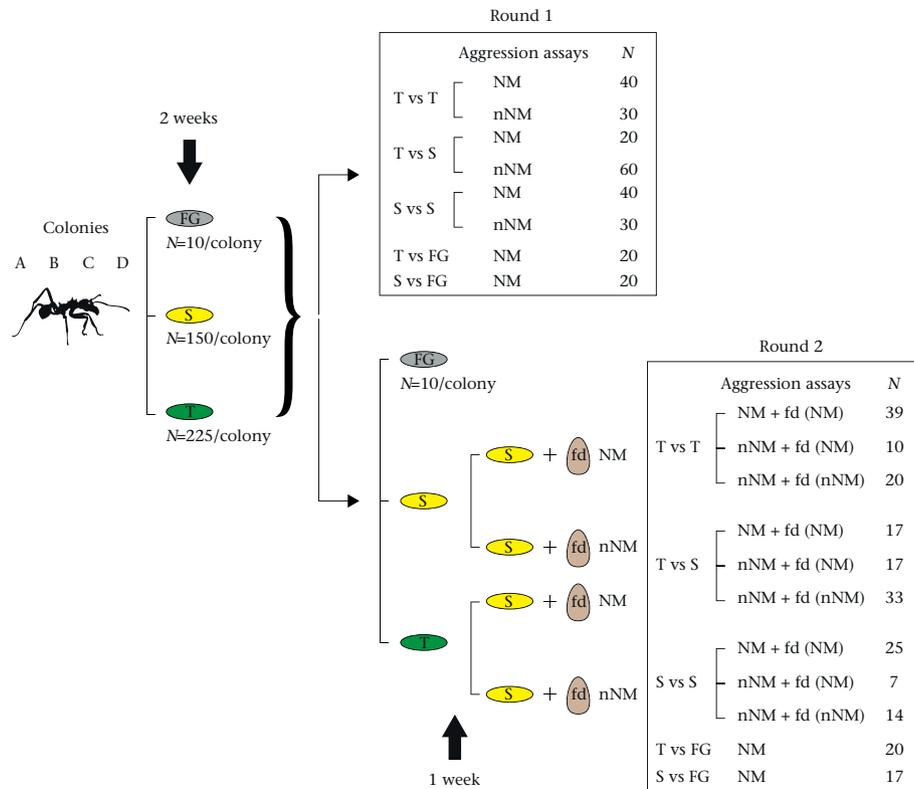


Figure 1. Experimental design. Large workers of *A. echinator* were collected from fungus gardens and kept on artificial sucrose or sucrose + tetracycline diets before aggression trials were performed after 2 weeks to compare workers on artificial diets with control workers on their normal fungus garden diet (Round 1). The guts of all experimental workers were then dissected to estimate absolute abundances of symbionts by qPCR and MiSeq of 16s rRNA, whereas remaining body parts (thoraxes and heads) were used for extractions of cuticular hydrocarbons and subsequent GC–MS analyses. In Round 2, a comparable set of workers from Round 1 that were still alive were transferred to a new experimental diet consisting of sucrose and faecal fluid from control workers of their own colony or one of the other (unrelated) colonies. In both rounds, the worker ants were also used in aggression trials to assess whether their social behaviours changed after the two diet treatments. FG: fungus garden ants; S: sucrose-treated ants; T: tetracycline-treated ants; fd: faecal droplets; NM: nestmate; nNM: non-nestmate.

Aggression Assays

We tested sucrose- and antibiotic-treated ants from Round 1, microbiota-remodelled ants from Round 2 and untreated ants against nestmates and non-nestmates from the original colonies (3–5 replicates/combination, total = 260 tests in Round 1 and 231 tests in Round 2; Fig. 1, see [Supplementary data set S1](#)). Assays consisted of dyadic encounters in petri dishes with clean filter paper on the bottom. For 2 min after first contact, an observer (blind with respect to ant treatment and original colony) used the software Etholog 2.25 (Otoni, 2000) to quantify the frequency and duration of biting, mandible opening, antennation and absence of contact (Appendix Fig. A3). For statistical analyses, we excluded the mandible opening behaviour (usually considered as aggressive) because pooling it with biting produced very similar results (data not shown). In addition, because keeping ants in the same petri dishes for 3 weeks resulted in a significant effect on aggression (binomial GLMM with ‘petri dish’ as fixed and ‘colony’ as random variables: $Z_{141} = 3.21$, $P = 0.001$), we considered only interactions between nestmates kept in different petri dishes (a total of 220 dyadic encounters for Round 1 and 157 for Round 2).

We analysed data in R using the packages lme4, car and multcomp (Bates, Mächler, Bolker, & Walker, 2015; Fox & Weisberg, 2011; Hothorn, Bretz, & Westfall, 2008), fitting generalized linear mixed models (GLMMs). Classifying biting as aggressive behaviour and antennal contact as nonaggressive allowed measures to be analysed as a single binomial response variable. For Round 1, the statistical model included biting versus antennation frequencies as the response variable, the factors nestmate relationship, diet treatment and their statistical interaction as fixed explanatory variables, and

colony affiliation of the paired experimental workers as random effect. The colony origin of experimental individuals was also included as a random effect. The model used for Round 2 included the same response variable as in the Round 1 model, and ‘faecal droplets’ as an additional fixed factor (two levels: from nestmates or non-nestmates). We tested the significance of fixed effects using the car function ‘Anova’. Where needed, we conducted post hoc planned contrast tests between groups of interest using the multcomp function ‘glht’, correcting alpha values for false discovery rate (FDR). Our R scripts are available in the [Supplementary material](#).

Effects of Diet Treatments on Survival

We analysed the effect of diet on survival with Cox proportional hazards models (with censoring), using the R package Survival (version 3.1.1, Therneau, 2015) function ‘coxph’, following assessment of proportional hazards using ‘cox.zph’. Survival in the two rounds was evaluated separately, because the diets, which were used as a cofactor affecting the mortality of ants, changed from Round 1 to Round 2. The other cofactors were colony origin and the tetracycline diet for Round 1, and colony origin and whether the ants had previously been treated with tetracycline or not in Round 2. Data were plotted using the survival and the GGally packages in R (Schloerke et al., 2014; Therneau, 2015).

DNA Extractions

Four to eight workers per treatment per colony that had been used in the aggression assays were ice anaesthetized and individually dissected in sterile phosphate-buffered saline. For each dissected

worker, pooled crop, midgut, hindgut, Malpighian tubules and fat body cells were stored at -20°C until DNA extractions. Malpighian tubules and fat body cells are not part of the intestinal system, but as in previous studies (Sapountzis, Nash, Schiøtt, & Boomsma, 2018; Sapountzis et al., 2015), we define the gut microbiomes broadly as the bacterial communities associated with the intestinal system consisting of the digestive tract and associated organs, also referred to as abdominal bacterial communities. For the DNA extractions, we homogenized the frozen samples using sterile pestles immediately after thawing in 200 μl ATL buffer supplemented with 20 μl proteinase K (Qiagen) using sterile pestles. Subsequently, glass beads 0.45 mm in diameter were added and tubes were vortexed for 30 s, after which the samples were incubated at 56°C overnight under constant agitation. DNA was extracted using the Qiagen Blood and Tissue kit, and all samples were eluted in 100 μl AE buffer.

16S rRNA qPCR Analyses

The gut microbiota of *A. echinator* normally consists of four predominant OTUs (operational taxonomic units, representing a cluster of bacterial 16S rRNA sequences of $\geq 97\%$ similarity; Van Borm et al., 2002; Sapountzis et al., 2015; Zhukova, Sapountzis, Schiøtt, & Boomsma, 2017). These belong to the genus *Wolbachia* (*WolAcro1*, including two strains: *wSinivictaA* and *wSinivictaB*) and the orders Entomoplasmatales (class: Mollicutes, OTUs *EntAcro1*, *EntAcro2* and *EntAcro10*) and Rhizobiales (class: Alpha-Proteobacteria, OTU *RhiAcro1*; Sapountzis et al., 2015). To monitor how the dietary treatment affected the communities, we screened individual worker guts and associated organs with qPCR (see detailed methods in Appendix Table A1) on the five most abundant OTUs (see Supplementary data set S2, procedures described in Sapountzis et al., 2015) with cycle threshold (C_T) means of replicated samples as measures of amplicon abundance. The elongation factor 1 alpha ($EF-1\alpha$) was used as a reference gene (Andersen, Yek, Nash, & Boomsma, 2015). Each run included two negative controls with no added template for each gene used. For analysis, we initially used a standard curve with PCR products in 10-fold dilution series of known concentration (fold change method) to calculate the PCR efficiency using the REST software (Pfaffl, Horgan, & Dempfle, 2002). Data were imported in R and expressed as $\Delta\Delta C_T$ values, i.e. as the fold change relative to the $EF-1\alpha$ control gene (Pfaffl, 2004), always using zero as reference. We used linear mixed models (LMM) with the $\Delta\Delta C_T$ values as response variable, 'diet treatment' (i.e. untreated, sugar-treated or tetracycline-treated) and 'experimental round' (i.e. Round 1 or 2) as fixed variables, and 'colony' (A, B, C or D) as a random variable. Tukey post hoc tests were performed to evaluate significant differences between groups. We used GLMMs to pair aggression data with distances based on $\Delta\Delta C_T$ fold change differences, or with differences in absolute bacterial cell numbers obtained from the qPCR data. All correlation analyses were performed in R using the packages lme4, vegan, effects, Rmisc and ggplot2 (Bates et al., 2015; Fox et al., 2017; Hope, 2013; Oksanen et al., 2017; Wickham, 2016).

16S rRNA MiSeq Sequencing and Analyses

To investigate whether novel OTUs appeared with the dietary treatment, we screened ant worker guts individually with 16S rRNA MiSeq sequencing. Amplicons were generated using the 515F/806R primers targeting the 16S rDNA V4 region (Caporaso et al., 2012). The PCR mix (20 μl) contained: 2.0 μl 10x AccuPrime PCR Buffer II (15 mM MgCl_2 , Life Technologies, Carlsbad, CA, U.S.A.), 0.15 μl AccuPrime Taq DNA Polymerase (2 units/ μl , Life Technologies), 1.0 μl of each primer (10 μM), 2 μl diluted template and water to a total of 20 μl . PCR incubation conditions were an initial activation of the hotstart polymerase at 94°C for 2 min, followed by 30–35

cycles of 94°C for 20 s, 56°C for 20 s and 68°C for 30 s and a final extension step at 68°C for 5 min. Samples were incubated at 70°C for 3 min, and then moved directly to ice to minimize hybridization between specific PCR products and short nonspecific amplicons. To add adapters and Index to DNA fragments, we added 2 μl of the diluted PCR products to the PCR mix to amplify ca. 458 bp fragments using different combinations of primers presented in Appendix Table A1. PCR was performed for 15 cycles and PCR products were purified using the Agencourt AMPure XP (Beckman Coulter, Brea, CA, U.S.A.) and quantified using Quant-iT dsDNA High-Sensitivity Assay Kit and Qubit fluorometer (Invitrogen, Carlsbad, CA, U.S.A.) to allow for dilution and mixing in equal concentrations before sequencing on an Illumina MiSeq.

Data [Genbank: SAMN04261407–SAMN04261536 and SAMN05362797–SAMN05362832] were analysed using mothur (Schloss et al., 2009) with two adjustments to the MiSeq standard operating procedure (SOP) due to slight differences in the sequencing protocol. First, after assembling the sequences we used the 'make.contigs' command in the software cutadapt (Martin, 2011) to remove the primer sequences, and continued with the mothur pipeline using the output; for the 'screen.seqs' command the start and the end were at 13 862 and 23 444 (see MiSeq SOP page). After filtering/processing the sequencing data and clustering at 97%, rarefaction tables were constructed using pseudoreplicate OTU data sets containing 1–272 000 sequences with 1000 iterations per pseudoreplicate, and the resulting curves were visualized in Microsoft Excel 2013. The final OTU Table was rarefied at 5000 reads after manual inspection of the rarefaction curves, which reduced the number of OTUs to 1500. We used the MiSeq data to calculate Canberra and Bray–Curtis distances, after which we used nonmetric multidimensional scaling (NMDS) to ordinate and visualize the effects. We used GLMs to pair aggression data with Bray–Curtis or Canberra distances calculated from the 16S rRNA MiSeq data. Correlation analyses were performed in R v3.2.3 using the lme4, vegan, effects, Rmisc and ggplot2 packages (Bates et al., 2015; Fox et al., 2017; Hope, 2013; Oksanen et al., 2017; Wickham, 2016). PERMANOVA tests were done in PAST using 1000 permutations and group differences were evaluated using Bonferroni-corrected multiple comparisons. We estimated the difference in variation between groups using the HOMOVA command (with Bonferroni-adjusted alpha values) implemented in mothur (Schloss et al., 2009).

Cuticular Hydrocarbon Analyses

CHCs were extracted by immersing the dissected heads and thoraxes of aggression test individuals, first in 150 μl HPLC-grade hexane and then in 150 μl HPLC-grade chloroform (chemicals from Sigma-Aldrich, Bornem, Belgium), both for 10 min under continuous agitation. The two extracts were mixed, and the solvent was evaporated at room temperature in a laminar flow cupboard. The dry extract was then dissolved in 30 μl hexane, of which 3 μl were injected in a Shimadzu QP2010 Ultra GC–MS (splitless injector mode). The injection temperature was 280°C and we used a DB-5 ms capillary column (30 m \times 0.25 mm \times 0.25 μm) with helium as the carrier gas at 1 ml/min. The oven temperature was held at 70°C for 1 min, then increased to 220°C at $25^{\circ}\text{C}/\text{min}$, and then to 325°C at $3^{\circ}\text{C}/\text{min}$, with a final hold for 15 min at 325°C . Our initial integration analysis of GC–MS runs detected 137 peaks, of which we selected 73 that had a relative abundance larger than 0.1% (Supplementary data set S3). Peak areas of cuticular compounds were integrated using R v3.1.0 (using package xcms, script available upon request) and normalized using a Z transformation (Aitchison, 1986). To compare CHC profiles between rounds, we used linear mixed models (LMM) with the Z-transformed abundance of each compound as the response variable, 'diet treatment' and

'experimental round' as fixed variables and 'colony' as a random variable. We considered linear hypotheses using the multcomp R package (Hothorn et al., 2008) function glht to evaluate differences between diet treatments in the same experimental rounds, and between the same diet treatments across rounds. All P values were corrected for FDR, given that we had conducted 73 separate tests per contrast. We used the package ade4 to perform coinertia analysis (Dolédec & Chessel, 1994) to check for correlations between CHCs (log-transformed peak areas) and qPCR measures ($\Delta\Delta C_t$ values) of the six bacterial OTUs considered (see also details below). In short, we generated two independent data matrices (either using the individual profiles or the pairwise differences for each trial), performed principal component analyses, and paired them using the coinertia analysis in a Monte Carlo test with 10 000 permutations.

RESULTS

Survival Analyses

During Round 1, mortality increased in tetracycline-treated workers (Cox proportional hazards model: $P < 0.001$), similar to a previous study (Sapountzis et al., 2015). However, this effect disappeared in Round 2, when all ants were fed on faecal droplets ($P = 0.329$; Appendix Fig. A1), suggesting that the harmful effect of tetracycline lasted only while it was administered to the ants. Colony origin also seemed to affect mortality in both rounds (Round 1: Colony B: $F = 0.38$, $P < 0.001$; Colony D: $F = 0.42$, $P < 0.001$; Round 2: Colony C: $F = 0.43$, $P = 0.017$; Colony D: $F = 0.46$, $P = 0.049$).

Round 1, Aggression Tests

When ants were isolated from their original colonies and fed on sterile sucrose diets with or without antibiotics (Fig. 1), the diet

treatment had a significant effect on aggression ($\chi^2_4 = 29.62$, $P < 0.001$), whereas being nestmates or non-nestmates did not make a significant difference for dyadic aggression ($\chi^2_1 = 1.64$, $P = 0.18$). We also found a significant statistical interaction between diet treatment and nestmate versus non-nestmate interactions ($\chi^2_2 = 6.9803$, $P = 0.031$) indicating that the strength of the diet effect differed depending on whether nestmates or non-nestmates interacted. The main effect of diet appeared to be mostly due to the high biting frequency between tetracycline-treated ants and their untreated former nestmates taken from the same fungus gardens, which was significantly higher than biting rates in all other nestmate trials (all $P < 0.05$; Fig. 2a, Appendix Table A2). However, for tetracycline-treated and sucrose-treated ants, the aggression levels between non-nestmates were not significantly different from those observed in nestmate trials (tetracycline-treated: $z = 0.365$, $P = 0.715$; Fig. 2a; sucrose-treated: $z = 1.284$, $P = 0.287$). This probably explains the significant overall interaction term between diet and (non)nestmate identities, also because the non-nestmate trials showed higher aggression levels than the nestmate trials in the sucrose- versus tetracycline-treated groups ($z = 2.375$, $P = 0.045$).

Round 1, Changes in Gut Bacterial Communities

As expected, the microbiomes of tetracycline-treated individuals were strongly affected, showing the lowest variance among OTUs because several of these were virtually eliminated; on the other hand, untreated individuals collected from fungus gardens showed the highest variance (HOMOVA: $P < 0.001$; Fig. 3). The gut bacterial communities of ants reared on fungus gardens or sucrose diets differed significantly from those of tetracycline-reared ants (PERMANOVA, multiple corrected Bonferroni comparisons: $P < 0.001$ and $P = 0.013$, respectively; Fig. 3), but their communities were not different from each other ($P = 0.053$). Furthermore, while diet had a

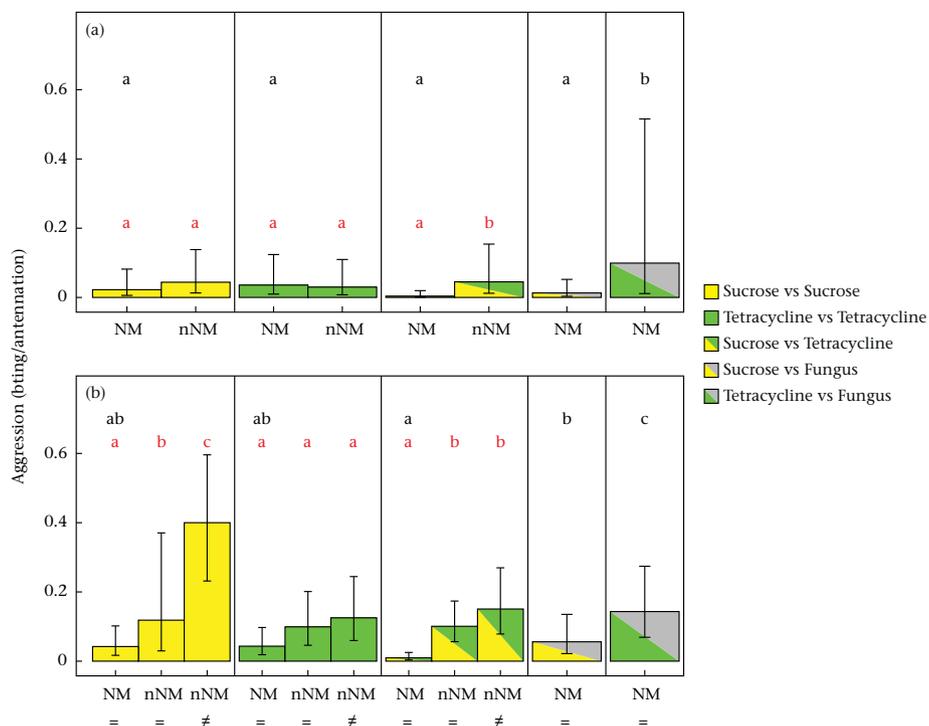


Figure 2. Effects of sucrose and tetracycline treatments on aggression in dyadic 1:1 encounters. (a) Round 1. (b) Round 2. Aggression was quantified as the mean rate of biting divided by the mean rate of antennation (baseline friendly exploration) with 95% upper and lower confidence limits as error bars. The same lowercase letters mark pairwise comparisons that were not significant on average, while different letters mark statistically significant differences ($P < 0.05$). Red letters refer to comparisons within treatment categories and black letters to comparisons across treatment categories. = and \neq symbols refer to diets with faecal fluid from the same or a different colony, respectively. NM: nestmate; nNM: non-nestmate.

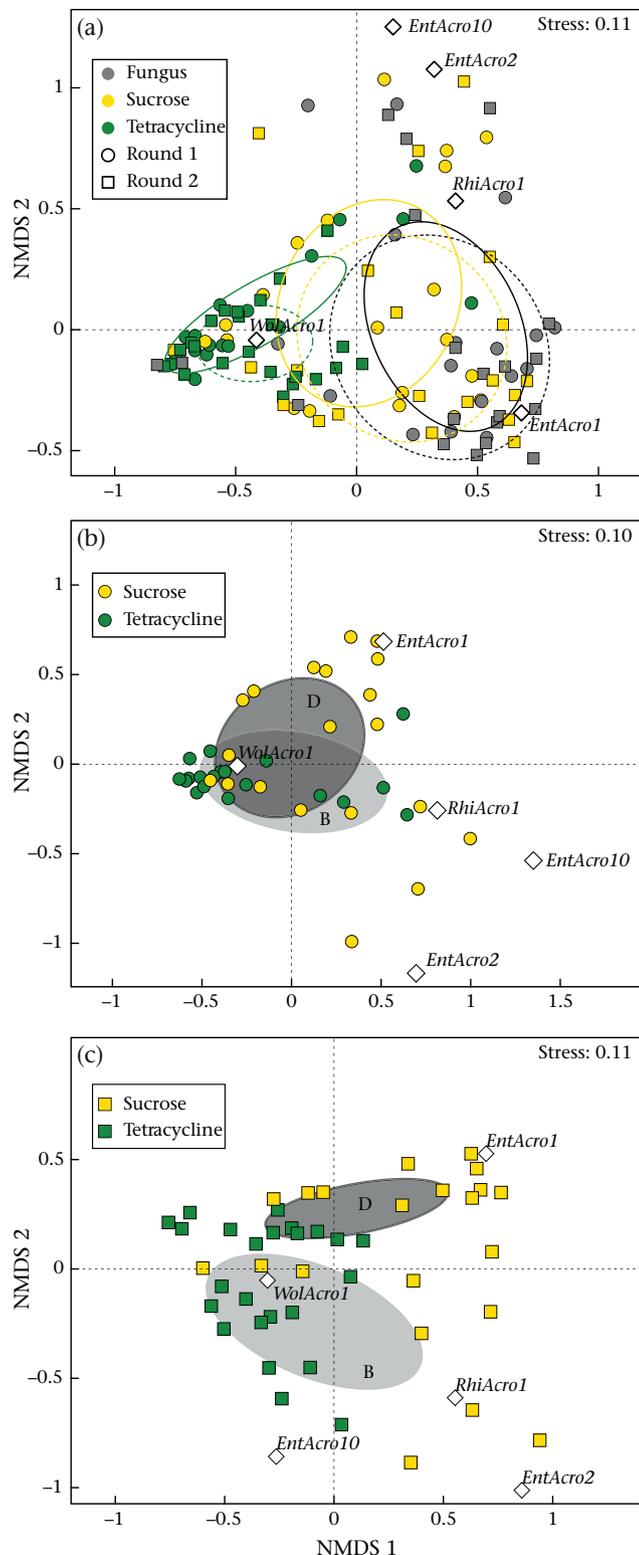


Figure 3. Nonmetric dimensional scaling (NMDS) ordinations of Bray–Curtis distances presenting the composition of gut bacterial communities (beta-diversity) of all 121 samples analysed in our study and using the rarefied MiSeq reads of the five most abundant gut bacterial OTUs (diamond shapes). (a) Ordination presenting samples from both rounds (drawn with different symbols) and all three treatments. Points and ellipses (95% CI) are colour-coded (black: control ants picked from fungus gardens; yellow: ants fed on sucrose; green: ants fed on tetracycline). Ellipses drawn with solid lines: Round 1; dashed lines: Round 2. (b) Sucrose-treated and tetracycline-treated ants from Round 1. (c) Sucrose-treated and tetracycline-treated ants from Round 2. In (b) and (c), grey ellipse areas show sample clustering based on the combination of

strong effect on the bacterial communities of treatment groups (PERMANOVA: $F_{2,53} = 9.162$, $P < 0.001$), colony identity was not important (PERMANOVA: $F_{3,53} = 1.758$, $P = 0.931$).

The most abundant OTUs of the *A. echinator* gut microbiota are *WolAcro1* (genus *Wolbachia*, including two strains: *wSinivictaA* and *wSinivictaB*) whose role in attine biology is unknown (Andersen, Boye, Nash, & Boomsma, 2012), the Mollicutes *EntAcro1*, *EntAcro2* and *EntAcro10*, which are the most abundant bacteria in the guts and surrounding tissues and are likely to be trophic mutualists (Sapountzis et al., 2015; Sapountzis, Zhukova, et al. 2018; Zhukova et al., 2017), and *RhiAcro1* (order Rhizobiales, class: Alpha-Proteobacteria), which has been suggested to complement the ants' nitrogen-limited diet (Sapountzis et al., 2015). When examining each of these OTUs individually, *EntAcro1* and *RhiAcro1* decreased substantially in the tetracycline-treated workers and moderately so in the sucrose-treated workers, while the *Wolbachia* OTU *wSinivictaB* was largely unaffected by diet (Fig. 4). *wSinivictaA* (present only in colony C, Supplementary data set S2), *EntAcro2* and *EntAcro10* (present, respectively, in nine and 20 of the 37 tested untreated workers taken from fungus gardens; Supplementary data set S2) increased slightly in sucrose-treated workers but decreased in the tetracycline-treated ants, although these effects were not significant. Our comparisons of microbial communities of heads, thoraxes and guts revealed that the most pronounced changes associated with different diets occurred in the guts and related abdominal tissues. In the gut samples a few distinct and abundant OTUs (mainly Mollicutes and Alpha-Proteobacteria) underwent major reductions in the antibiotics diet treatment, while the head and thorax samples showed minor effects on a larger range of other OTUs (including Alpha-Proteobacteria, Actinobacteria, Sphingobacteria, Beta-Proteobacteria; Appendix Fig. A2). Visual inspection of Fig. A2 suggested that the tetracycline and sugar diets affected (strongly and moderately, respectively), *RhiAcro1* and the Mollicutes OTUs (*EntAcro1*, *EntAcro2* and *EntAcro10*) in the gut, similar to findings in a previous study (Sapountzis et al., 2015). We did not detect any treatment-related major differences in *WolAcro1* abundances in the gut, and neither did any of the major taxa present in the head and thorax tissues show significant differences. The inference that the major effects in microbiota due to diet treatments occur in the gut was confirmed by qPCR: in gut tissues, differences in the abundant bacterial taxa due to diet treatments were always highly significant and involved several-fold changes (*EntAcro1*: $\chi^2_4 = 18.86$, $P < 0.001$; *wSinivictaA*: $\chi^2_4 = 80.59$, $P < 0.001$; *wSinivictaB*: $\chi^2_4 = 41.99$, $P < 0.001$; *RhiAcro1*: $\chi^2_4 = 26.82$, $P < 0.001$). However, in the heads and thoraxes, of the three most abundant taxa (the joint average abundance of *wSinivictaA*, *wSinivictaB* and *ActAcro1* in the guts of control ants taken from fungus gardens was 46–57% of all OTUs present), only the less than one-fold differences in *wSinivictaB* were significant between treatments ($\chi^2_4 = 96.55$, $P < 0.001$), while the differences in *wSinivictaA* and *ActAcro1* were either marginally or not significant (*wSinivictaA*: $\chi^2_4 = 10.43$, $P = 0.033$; *ActAcro1*: $\chi^2_4 = 0.93$, $P = 0.919$).

Round 2, Aggression Tests

During Round 2, we compared aggression across sucrose-treated pairs, tetracycline-treated pairs and sucrose- versus tetracycline treated pairs. Aggression was low in nestmate encounters of ants fed with nestmate faecal droplets, higher in non-nestmate encounters of ants fed with nestmate faecal droplets, and highest in encounters of

round 1 feeding treatment and round 2 faecal droplet restoration treatment (light grey ellipse: samples originating from ants fed with faecal droplets from workers from colony B; dark grey ellipse: samples originating from ants fed with faecal droplets from workers from colony D; see methods for details).

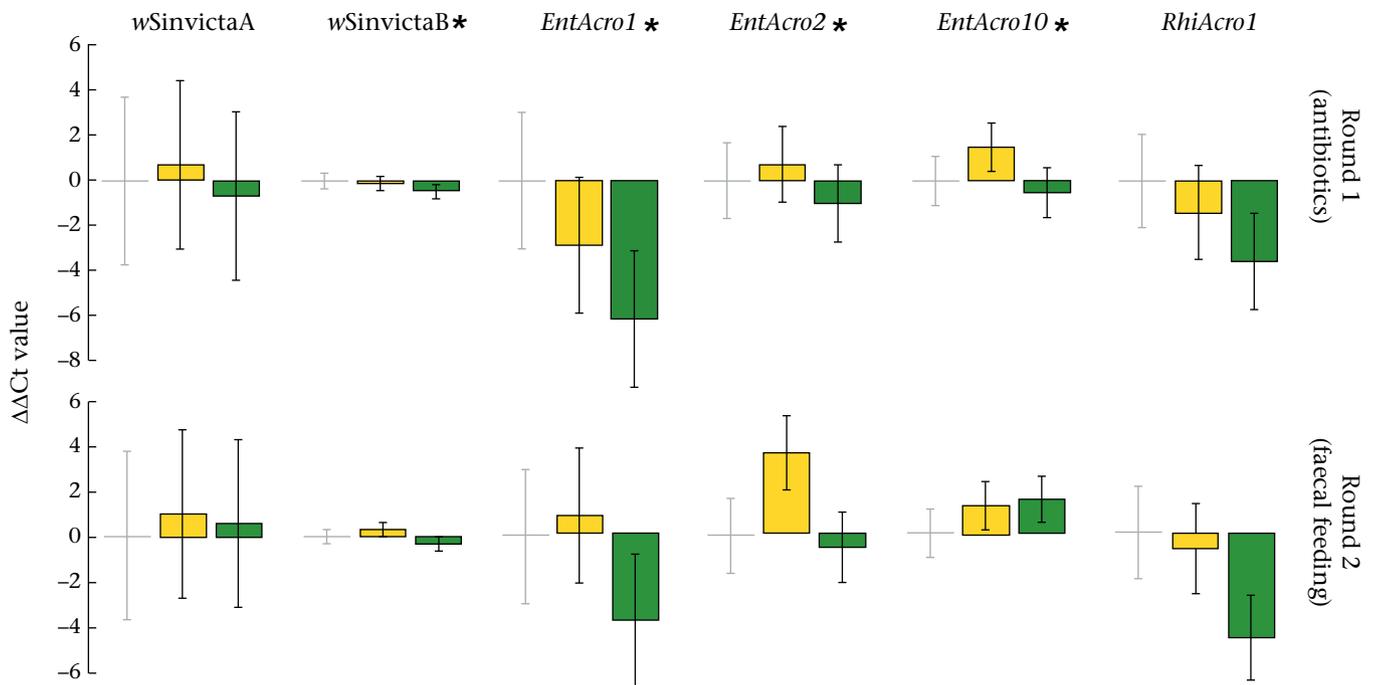


Figure 4. Changes in qPCR-estimated bacterial titres expressed as fold changes for the six most abundant bacterial OTUs after experimental Rounds 1 and 2 on a sucrose diet (yellow bars) and a tetracycline diet (green bars). Counts were normalized relative to EF-1a copies to produce unbiased fold changes ($\Delta\Delta\text{Ct}$) using the samples originating from ants reared on a normal fungal diet as reference level (grey $y = 0$ values), all with 95% confidence intervals. Asterisks indicate significant differences ($P < 0.05$) between treatment and control groups in round 2 (there were no significant differences between treatments in round 1).

non-nestmate ants each fed with non-nestmate faecal droplets (Fig. 2b). In particular, the highest aggression levels appeared in encounters between non-nestmate sucrose-treated ants fed with faecal droplets from different colonies ($z_{85} = 2.05$, $P = 0.041$; Fig. 2b, Appendix Table A2). In contrast, tetracycline-treated pairs always showed low aggression (Fig. 2b) regardless of whether they were fed on faecal droplets of workers from the same or a different colony. Also, confrontations between sucrose-treated versus tetracycline-treated ants showed low aggression levels, similar to confrontations between tetracycline-treated pairs. We found a significant statistical interaction between faecal droplet supplementation and diet treatment ($\chi^2_2 = 7.57$, $P < 0.05$), but not between diet treatment and nestmate versus non-nestmate confrontations ($\chi^2_2 = 3.36$, $P = 0.18$; Fig. 2b, Appendix Table A2). All three main effects were significant (faecal droplet supplementation or not: $\chi^2_1 = 7.99$, $P < 0.05$; initial diet treatment: $\chi^2_4 = 36.97$, $P < 0.001$; confrontations between nestmates versus non-nestmates: $\chi^2_1 = 5.2437$, $P < 0.05$).

Round 2, Changes in Gut Bacterial Communities

The NMDS ordination suggested that the gut bacterial communities of tetracycline-treated workers before and after faecal droplet feeding were not significantly different. However, after faecal droplet feeding, the gut bacterial communities of sugar-treated ones were closer to those of untreated ones sampled from original fungus gardens in Round 1, suggesting a partial shift backwards towards the original communities (Fig. 3). The gut bacterial samples of sugar-treated workers exhibited a clear separation depending on which type of faecal droplets (from nestmates or non-nestmates) they were fed with (PERMANOVA, diet treatment: $F_{1,43} = 23.17$, $P < 0.001$; faecal droplets: $F_{1,43} = 12.10$, $P < 0.001$; interaction: $F_{1,43} = 5.04$, $P = 0.004$), whereas there was no such effect in the tetracycline-treated group (Fig. 3). The qPCR data showed that tetracycline-treated ants undergoing faecal droplet feeding experienced an increase in all the most abundant OTUs (except for *EntAcro2* and

RhiAcro1, whose levels were further reduced); sucrose-treated ants showed an increase of all six gut bacterial OTUs examined (Fig. 4), but only changes in *EntAcro1*, *EntAcro2*, *EntAcro10* and *wSinivictaB* were significant (respectively: $t = -2.69$, $P = 0.008$; $t = -2.02$, $P = 0.044$; $t = -5.32$, $P < 0.001$; $t = -5.95$, $P < 0.001$).

Associations between Changes in Endosymbiont Abundance and Aggression

To identify which of the OTUs were associated with worker aggression when bacterial titres were manipulated, we regressed the observed aggression measurements between nestmate test pairs on the differences in abundance of their gut bacteria (Fig. 5). This analysis (based on 1500 MiSeq OTUs; Bray–Curtis distances between pairs) confirmed that workers were generally more aggressive in pairwise tests when experimental manipulation had changed their gut microbial community more (binomial GLMM with ‘genetic distance’ and ‘experimental round’ as fixed variables and ‘colony’ as random variable: $z = 4.91$, $P < 0.001$). For the six separate OTUs, we used the qPCR data, regressing observed pairwise aggression against OTU-specific $\Delta\Delta\text{Ct}$ values (Fig. 5). This analysis showed that aggression was not affected by changes in the titres of one of the two *Wolbachia* strains (Fig. 5; *wSinivictaA*: $z = -0.66$, $P = 0.509$) and the *EntAcro2* symbiont ($z = -0.872$, $P = 0.383$), but that there was a significantly negative correlation with *wSinivictaB* ($z = -3.75$, $P < 0.001$) and significantly positive correlations for *EntAcro1* ($z = 2.10$, $P = 0.035$), *EntAcro10* ($z = 6.09$, $P < 0.001$) and *RhiAcro1* ($z = 5.85$, $P < 0.001$). When splitting the data for each of the two rounds, changes in *EntAcro1* abundances showed a significant positive correlation with changes in aggression in Round 1, while this was so for *EntAcro10* in Round 2 (Supplementary data set S4). Only differences in abundance of *RhiAcro1* had a significant effect on aggression in both rounds (Fig. 5; Supplementary data set S4; Round 1: $z = 3.25$, $P < 0.001$; Round 2: $z = 4.17$, $P < 0.001$), suggesting that manipulation of Rhizobiales titres has consistent consequences for aggression

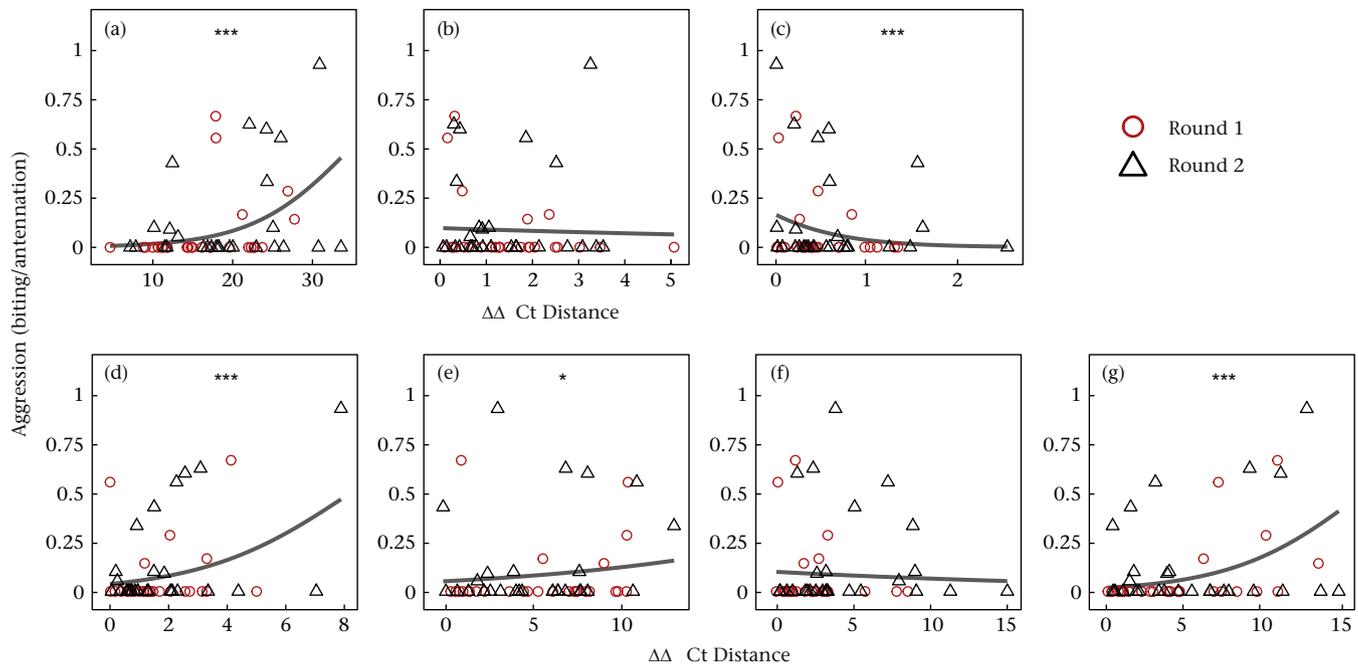


Figure 5. Association between aggression levels and $\Delta\Delta$ Ct abundance differences due to diet treatment for all six abundant bacterial taxa in the gut microbiomes (pooled) and for the six OTUs separately as estimated with GLMM regressions. (a) All six abundant OTUs pooled, (b) *wSinivictaA*, (c) *wSinivictaB*, (d) *EntAcro10*, (e) *EntAcro1*, (f) *EntAcro2* and (g) *RhiAcro1*. All data from experimental rounds 1 and 2 are pooled. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

towards nestmates (see [Supplementary data set S4](#)). The presented results are based on biting only, but very similar results were obtained when both biting and mandible opening were treated as aggressive behaviours (data not shown).

CHC Profiles are Associated with Diet

Compared to untreated individuals from original fungus gardens, sucrose- and tetracycline-treated workers (data from Round 1 and Round 2 combined) had strong reductions of 4-oxo-octanoic and 4-oxo-decanoic acids in their CHC profiles (LMMs with 'diet' and 'round' as fixed variables and 'colony' as random variable: FDR-corrected P values for multiple comparisons for 4-oxo-octanoic acid: untreated versus sucrose-treated: $t = 15.20$, $P < 0.001$; untreated versus tetracycline-treated: $t = 15.01$, $P < 0.001$; 4-oxo-decanoic acid: untreated versus sucrose-treated: $t = -13.10$, $P < 0.001$; untreated versus tetracycline-treated: $t = -13.66$, $P < 0.001$). Changes to these two compounds were significant not only in overall comparisons, but also in all pairwise comparisons among treatment groups ([Supplementary data set S4](#)), while changes in two other compounds, the long-chain linear hydrocarbons $n\text{-C}_{36}$ and $n\text{-C}_{40}$, were only significant in comparisons between workers treated with antibiotics and the fungus-garden control workers from their home colonies.

Considering the overall association between cuticular chemical profiles and the abundances of symbiont OTUs as measured with qPCR, we found a significant association between them (co-inertia analysis: $RV = 0.143$, $P < 0.001$; Fig. A4). When looking at each OTU separately, the strongest pattern was an association between *EntAcro1* and *RhiAcro1* and 4-oxo-octanoic and 4-oxo-decanoic acids. Even after we analysed the data without these two acids, we recovered a significant overall correspondence between chemical profiles and qPCR gut bacterial abundances ($RV = 0.141$, $P < 0.001$). However, we could not find a significant association between CHC profile and gut microbiota when we used chemical distance and the difference in qPCR counts between individuals in aggression pairs ($RV = 0.122$, $P = 0.707$). Altogether, this suggests that bacterial abundances can

explain some of the colony level changes in CHC profile that are likely to mediate aggression induced by our manipulative diets, but that aggression in specific dyadic encounters between worker ants cannot be predicted by CHC profiles and bacterial titres.

DISCUSSION

Diet and Antibiotics Treatment Affect Gut Microbial Communities

The gut microbial communities of tetracycline-treated ants underwent substantial changes relative to untreated nestmate ants subjected to control experiments (two OTUs decreased strongly, three slightly and one was not affected; Fig. 4), whereas the microbiomes of sucrose-treated ants exhibited relatively milder shifts (three OTUs decreased slightly, two increased slightly and one was not affected). These results show that while the antibiotic treatment suppressed most of the endosymbionts in the guts and associated organs of ant workers, the sterile sucrose diet had only a mild effect on relative abundances of OTUs.

Feeding workers with conspecific faecal droplets only partially restored the original gut microbial communities of sucrose- and tetracycline-treated ants, suggesting that the elimination of some strains such as *RhiAcro1* (and *EntAcro1*, but only in tetracycline treated ants) was irreversible. This may imply that some bacteria need to be established during larval development or early adult life and cannot be reintroduced later. In addition, previously latent OTUs (e.g. *EntAcro10*) increased in prevalence, which may have prevented the original OTUs from recolonizing the tissues/organs from which they were eliminated (cf. [Anderson, Rodrigues, Mott, Maes, & Corby-Harris, 2015](#); [Salem, Florez, Gerardo, & Kaltentpoth, 2015](#)). We also cannot exclude that the tetracycline effect lasted for some time even after its administration was suspended (1 week before the preparation of the corresponding microbial DNA samples), potentially interfering with the faecal droplet-mediated microbial gut recolonization.

Although we provide evidence that some bacterial taxa were more affected by our experimental food treatments than others,

amplicon sequencing often does not allow bacterial strains to be distinguished because closely related strains have identical 16S sequences (Andersen et al., 2012; Engel, Stepanauskas, & Moran, 2014; Kuo, 2015). Thus, we cannot exclude that effects of gut bacteria on behaviour may be driven by the interaction of multiple bacterial strains with different metabolic potential that were captured under the same 97% identity OTUs. Further research should thus implement methods allowing higher resolution.

Diet Treatments Affect Ant Cuticular Chemical Profiles

Our experiments showed that diet treatments significantly affected ant CHC profiles, and further analyses revealed a correlation between CHC profiles and bacterial abundances. However, we acknowledge that the experimental removal of gut bacteria probably introduced confounding effects on the wellbeing and survival of the ants because (1) antibiotics may have a direct detrimental effect on mitochondrial functionality (Moullan et al., 2015) and (2) at least some of the gut bacteria will probably have metabolic roles in covering direct energy needs or in the production of metabolites that are absent in host diets (Feldhaar et al., 2007; de Souza, Bezier, Depoix, Drezén, & Lenoir, 2009; Russell et al., 2009; Hu et al., 2018) as recently suggested for *EntAcro1* (Sapountzis, Zhukova, et al. 2018). More work will therefore be needed to unravel direct causation and collateral effects.

With these caveats in mind, we found consistent and significant decreases in two acids (4-oxo-octanoic and 4-oxo-decanoic) that are known to be specific products of metapleural gland secretion (Ortius-Lechner, Maile, Morgan, & Boomsma, 2000; Larsen, Fouks, Bos, d'Ettorre, & Nehring, 2014) and in two long-chain linear alkanes (n-C₃₆ and n-C₄₀) in the tetracycline-treated individuals. While the latter probably originate as cuticular hydrocarbons of unknown function and production costs, the former have disease defence functions and are known to be metabolically costly to produce (Poulsen & Boomsma, 2003). The observed changes in chemical profiles may thus have been affected either directly by symbiotic gut bacteria or, more likely, indirectly because of compromised host metabolism and deficiency in resources to produce these compounds. This inference is supported by the fact that CHCs are synthesized in the oenocytes (Ringo et al., 2011), which are the overall centre of intermediate metabolism in ants (Arrese & Soulages, 2010) and are heavily colonized by *EntAcro1* bacteria, recently shown to probably complement the *Acromyrmex* metabolism (Sapountzis et al., 2015; Sapountzis, Zhukova et al., 2018).

Tetracycline treatment may have affected not only the gut microbes, but also the microbiota on the ants' cuticle, particularly the *Pseudonocardia* actinobacterial symbionts of *Acromyrmex* (Andersen et al., 2013, 2015), again most likely as an indirect consequence of compromised metabolism affecting nutrient provisioning of cuticular biofilms. In *Drosophila*, cuticular microbes have been hypothesized to affect CHC profiles because compounds embedded in the cuticular wax layer may function as carbon sources and be enzymatically degraded (Ringo et al., 2011). Previous research on *Pogonomyrmex* ants showed that topical antibiotic administration can indeed alter CHC profiles of workers, consistent with a possible role of surface microbes in determining CHC profiles (Dosmann et al., 2016). However, the same kind of antibiotic treatment that we used here did not affect CHC profiles when applied on *A. s. subterranean* (de Souza et al., 2013). Other research suggests that innate immune responses may also affect CHC profiles, both in *Drosophila* (Ryu, Ha, & Lee, 2010) and in the honey bee, *Apis mellifera* (Richard, Aubert, & Grozinger, 2008). Multiple factors are therefore conceivably involved and likely to interact with each other, so disentangling the implications for CHC profiles will require extensive further work.

Our correlational evidence for dietary treatments affecting gut bacteria, reflecting a mostly unknown network of causation factors,

should also prompt further evaluation of other covariates that could play a role. Across insect taxa, symbiotic bacteria produce a plethora of volatile semiochemicals (Davis, Crippen, Hofstetter, & Tomberlin, 2013; Ezenwa & Williams, 2014), and studies on *Drosophila* suggest that compounds derived from the metabolism of gut microbes can also mediate interactions between host individuals (Lizé et al., 2014; Venu, Durisko, Xu, & Dukas, 2014). Superorganismal ant colonies may have integrated the bacterial metabolism in their chemical-based social dynamics, and ants may thus rely to an unknown extent on a social communication system based on chemicals produced by the colony level community of endosymbiotic bacteria. If these were sufficiently distinct, this might imply that artificial removal of endosymbionts will handicap social functionality by triggering either inappropriate aggression or a lack of aggression to certain challenges, consistent with the effects we found in our study. Accordingly, we cannot exclude that the altered behaviour observed in our aggression tests may at least partially depend on volatile semiochemicals related to bacterial metabolism and that social handicaps may not be completely reducible to CHCs on the cuticle. This complicates recommendations for future experiments. It will be important to find ways to do nestmate recognition assays in which microbiota-free and microbiota-remodelled interacting individuals are not exposed to nonvolatile cuticular social cues. However, taking into account that ants and endosymbionts are both likely to have their own genetically and environmentally mediated volatiles and CHCs will tend to make experimental designs highly complex when all factors and their interactions are to be unravelled.

Diet Suppresses Microbes, Potentially Affecting Social Interactions

Encounters between antibiotic-treated and untreated nestmates in Round 1 produced the highest aggression levels, suggesting that the antibiotic treatment affected chemical cues that are relevant for social interactions. Aggression due to changed diet clearly gave a steeper response than aggression due to nestmate status. Sucrose-treated ants gave relatively straightforward behavioural responses after the faecal droplet treatment, but tetracycline-treated ants tended to produce less clear contrasts (Fig. 2a). Nevertheless, it can be inferred that tetracycline-treated workers were less aggressive and were attacked more often. In fact, aggression among tetracycline-treated ants was constantly lower than in all other groups. However, tetracycline-treated versus untreated workers showed similar aggression levels across the two rounds, suggesting that untreated ants attacked treated ants and not the other way around. If tetracycline-treated workers were more aggressive, aggression in tetracycline-treated pairs should have been higher. Tetracycline-treated ants showed low levels of aggression in the second round, which was as expected because their gut microbiota were not completely re-established, consistent with behaving similarly to ants tested in the first round. Finally, tetracycline-treated versus sucrose-treated pairs in the second round showed an intermediate level of aggression between sucrose versus sucrose (highest) and tetracycline versus tetracycline pairs (lowest), suggesting that sucrose-treated workers attack tetracycline-treated workers, and not the other way round. Although these results are only correlational, they suggest that tetracycline-treated workers received more aggression than they expressed themselves.

As mentioned above, tetracycline is likely to compromise the functionality of mitochondria and some specific tissues (Moullan et al., 2015), which makes the effect of the antibiotic itself on behaviour difficult to disentangle from the effects of induced changes in microbial communities, consistent with the higher mortality rates in antibiotic-treated ants in Round 1 (Appendix Fig. A1; Sapountzis et al., 2015). Nevertheless, even if correlative, the connection

between observed aggression and gut bacterial abundances was highly significant. A possible interpretation of the increased aggression levels towards nestmates with suppressed gut microbes and abnormal odours (CHC profiles) may be that these workers are perceived as abnormal or infected. If so, it might be understandable that fungus garden workers would adaptively use aggression to push such deviating workers into foraging and defensive tasks outside or towards the periphery of the colony to minimize the spread of putative infections within the colony. On the other hand, lack of aggression between antibiotic-treated non-nestmates also suggests that the absence of symbiotic microbes makes such microbially compromised workers unsuitable to patrol territorial boundaries.

The Impact of Microbiota Restoration on Social Interactions

After faecal droplet administration, dyadic encounters between nestmates revealed only moderate and nonsignificant aggression levels, barely higher than those observed in Round 1. While these slightly higher responses may have been an effect of the relatively longer separation of the experimental ant groups from their source colonies (3 weeks instead of 2 weeks in petri dishes), the lack of significant differences between experimental rounds is also consistent with the microbial gut communities only having become partly restored. In non-nestmate aggression assays, pairs of tetracycline-treated and tetracycline-treated versus sucrose-treated ants also showed low aggression levels overall. Sucrose-treated non-nestmate dyads were more aggressive than nestmate dyads, and we could attribute this to a colony level difference in cuticular hydrocarbon profiles. In contrast, aggression was significantly higher in sucrose-treated pairs when interacting individuals were fed with faecal droplets from different colonies, strongly suggesting an effect of microbes on nestmate discrimination. This seems to imply that, while bacterial communities were indeed partly restored, it was important that this restoration involved the colony's standard mix of microbes and fungal enzymes known to be transferred via faecal fluid (Kooij et al., 2014; Poulsen & Boomsma, 2005; Schiøtt, Rogowska-Wrzęsinska, Roepstorff, & Boomsma, 2010). However, the partial restoration of gut microbiota suggests that the effect of tetracycline cannot solely explain the observed aggression in Round 1. Even if there were confounding tetracycline effects, the bacterial suppression and partial restoration accounted to a significant degree for the observed aggression patterns.

Conclusions

In this study, we developed an integrative approach to explore the role of gut microbial symbionts in the social dynamics of ant colonies with complex division of labour and obligate symbiotic dependence on a coevolved fungal cultivar. Dietary changes, which resulted in experimental suppression and remodelling of the ant gut microbiota, affected both the cuticular chemicals that ants displayed as socially relevant recognition cues and the social behaviours expressed. Our findings suggest that the observed effects on mutual aggression between individual workers may be related to changes in the abundances of two symbiotic bacterial OTUs, the previously characterized major gut bacterial taxa in leaf-cutting ants *EntAcro1* and *RhiAcro1* (Andersen et al., 2013; Sapountzis, Zhukova et al., 2018; Sapountzis et al., 2015). The observed differences in the resilience or re-establishment of these two symbionts may reflect differences in their tissue specificity: *EntAcro1* is abundant in the lumen and thus easily removed by antibiotics, while *RhiAcro1* forms a biofilm in the hindgut and should thus be more resistant to antibiotics, but also harder to reestablish (Sapountzis et al., 2015). Further research will be needed to address the mechanisms underlying the link between the changes in relative abundance of these symbionts and the expression

of a variety of social behaviours, not just aggression. Our results suggest that altered microbial communities may result in chemical profiles that are not recognized as those of nestmates, or that microbiota-free or remodelled ants may be recognized as sick and unsuitable for central colony functions such as brood nursing or garden hygiene. Social withdrawal of unhealthy workers has been demonstrated for two ant species (Bos, Lefèvre, Jensen, & d'Ettorre, 2012; Heinze & Walter, 2010), and it is thus conceivable that ostracism of sick ants is driven by changes in CHCs or other volatile compounds that are important in communication. Regardless, our findings provide evidence that gut bacterial symbionts may be involved in multiple forms of social discrimination in ants so that the concept of a healthy colony level gut microbiome may have similar far-reaching implications as has been repeatedly suggested for humans and farm animals (Bourtzis & Miller, 2003; Dubilier, Bergin, & Lott, 2008; Hara et al., 2013; Jha & Berrocoso, 2016; Kamada, Chen, Inohara, & Núñez, 2013; Kanji et al., 2018).

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Supplementary material

Supplementary material related to this article can be found at <https://doi.org/10.1016/j.anbehav.2018.12.017>.

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Appendix

Table A1
Primers used for library preparation of the MiSeq sequencing for the main diet experiment

Primer	Sequence
Forward	
MS_515f_F1	AATGATACGGCGACCACCGAGATCTACACAACCAACGTCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGAGTCCAGCMGCCGCGGTAA
MS_515f_F2	AATGATACGGCGACCACCGAGATCTACACAAGAGCTCTCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCGGGAGTCCAGCMGCCGCGGTAA
MS_515f_F3	AATGATACGGCGACCACCGAGATCTACACAGATAAGTCTCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTGGCCGGGAGTCCAGCMGCCGCGGTAA
MS_515f_F4	AATGATACGGCGACCACCGAGATCTACACAACCACTCTCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGAATGGCCGGGAGTCCAGCMGCCGCGGTAA
MS_515f_F5	AATGATACGGCGACCACCGAGATCTACACCTAGCGATCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTGAATGGCCGGGAGTCCAGCMGCCGCGGTAA
MS_515f_F6	AATGATACGGCGACCACCGAGATCTACACGCTCTGCTCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGGAGTCCAGCMGCCGCGGTAA
MS_515f_F7	AATGATACGGCGACCACCGAGATCTACACGGCGTCTCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCGGGAGTCCAGCMGCCGCGGTAA
MS_515f_F8	AATGATACGGCGACCACCGAGATCTACACGCTAGTCTCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTGGCCGGGAGTCCAGCMGCCGCGGTAA
MS_515f_F9	AATGATACGGCGACCACCGAGATCTACACTGCTGCTCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGAATGGCCGGGAGTCCAGCMGCCGCGGTAA
MS_515f_F10	AATGATACGGCGACCACCGAGATCTACACTAGATCTGCTCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTGAATGGCCGGGAGTCCAGCMGCCGCGGTAA
Reverse	
MS_806r_R1	CAAGCAGAAGACGGCATAACGAGATAACCAACGGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGAACGGACTACHVGGGTWCTAAT
MS_806r_R2	CAAGCAGAAGACGGCATAACGAGATAACCAACGGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCCGGGAGGACTACHVGGGTWCTAAT
MS_806r_R3	CAAGCAGAAGACGGCATAACGAGATACTGCTCTGCTCGTGGGCTCGGAGATGTGTATAAGAGACAGACGCTCTAACGGACTACHVGGGTWCTAAT
MS_806r_R4	CAAGCAGAAGACGGCATAACGAGATATACAGGATCTCGTGGGCTCGGAGATGTGTATAAGAGACAGAAACGCTCTAACGGACTACHVGGGTWCTAAT
MS_806r_R5	CAAGCAGAAGACGGCATAACGAGATATGATTAGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTGGGAAACGCTCTAACGGACTACHVGGGTWCTAAT
MS_806r_R6	CAAGCAGAAGACGGCATAACGAGATCAACCTCTGCTCGTGGGCTCGGAGATGTGTATAAGAGACAGAACGGACTACHVGGGTWCTAAT
MS_806r_R7	CAAGCAGAAGACGGCATAACGAGATCAGAATATGCTCTGCTGGGCTCGGAGATGTGTATAAGAGACAGCCGGGAGGACTACHVGGGTWCTAAT
MS_806r_R8	CAAGCAGAAGACGGCATAACGAGATCCATGCGCGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGACGCTCTAACGGACTACHVGGGTWCTAAT
MS_806r_R9	CAAGCAGAAGACGGCATAACGAGATCCGCTAGGGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGAAACGCTCTAACGGACTACHVGGGTWCTAAT
MS_806r_R10	CAAGCAGAAGACGGCATAACGAGATCGCATCATGCTCTGCTGGGCTCGGAGATGTGTATAAGAGACAGTGGGAAACGCTCTAACGGACTACHVGGGTWCTAAT
MS_806r_R11	CAAGCAGAAGACGGCATAACGAGATGAGGATAGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGAACGGACTACHVGGGTWCTAAT
MS_806r_R12	CAAGCAGAAGACGGCATAACGAGATGCTGGTCACTCGTGGGCTCGGAGATGTGTATAAGAGACAGCCGGGAGGACTACHVGGGTWCTAAT
MS_806r_R13	CAAGCAGAAGACGGCATAACGAGATGGAGGCTCGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGACGCTCTAACGGACTACHVGGGTWCTAAT
MS_806r_R14	CAAGCAGAAGACGGCATAACGAGATGTAAGAAGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGAAACGCTCTAACGGACTACHVGGGTWCTAAT
MS_806r_R15	CAAGCAGAAGACGGCATAACGAGATGTTAAGTCTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTGGGAAACGCTCTAACGGACTACHVGGGTWCTAAT
MS_806r_R16	CAAGCAGAAGACGGCATAACGAGATTCGCTGAGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGAACGGACTACHVGGGTWCTAAT
MS_806r_R17	CAAGCAGAAGACGGCATAACGAGATTGAATGGCTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCCGGGAGGACTACHVGGGTWCTAAT
MS_806r_R18	CAAGCAGAAGACGGCATAACGAGATTGATCTCAGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGACGCTCTAACGGACTACHVGGGTWCTAAT
MS_806r_R19	CAAGCAGAAGACGGCATAACGAGATTGGTCTGCTCGTGGGCTCGGAGATGTGTATAAGAGACAGAAACGCTCTAACGGACTACHVGGGTWCTAAT
MS_806r_R20	CAAGCAGAAGACGGCATAACGAGATTGTTGACGCTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTGGGAAACGCTCTAACGGACTACHVGGGTWCTAAT

Table A2
Aggression tests: post hoc planned contrasts among tests between treatment groups

Round	Comparisons	Estimate	SE	z	P	
1	NM S vs S	nNM S vs S	-0.6976	0.5431	-1.284	0.287
1	NM S vs T	nNM S vs T	-24.867	10.472	-2.375	0.046
1	NM T vs T	nNM T vs T	0.1853	0.5078	0.365	0.715
1	NM S vs S	NM S vs T	17.638	10.708	1.647	0.185
1	NM S vs S	NM T vs T	-0.4822	0.5310	-0.908	0.430
1	NM S vs S	NM T vs FG	-15.691	0.4628	-3.391	0.004
1	NM S vs S	NM S vs FG	0.5509	0.7061	0.780	0.472
1	NM T vs T	NM S vs T	22.460	10.454	2.149	0.069
1	NM T vs T	NM T vs FG	-10.869	0.3990	-2.724	0.021
1	NM T vs T	NM S vs FG	10.331	0.6674	1.548	0.198
1	NM S vs T	NM S vs FG	-12.129	11.444	-1.060	0.376
1	NM S vs T	NM T vs FG	-33.329	10.120	-3.293	0.004
1	NM S vs FG	NM T vs FG	-21.200	0.6146	-3.449	0.004
2	NM S vs S— Same FD	nNM S vs S— Same FD	-11.145	0.4867	-2.290	0.035
2	NM S vs S— Same FD	nNM S vs S— Different FD	-27.164	0.5421	-5.011	8.65e-06
2	nNM S vs S— Same FD	nNM S vs S— Different FD	-16.019	0.5664	-2.828	0.010
2	NM T vs T— Same FD	nNM T vs T— Same FD	-0.8886	0.4715	-1.885	0.080
2	NM T vs T— Same FD	nNM T vs T— Different FD	-11.513	0.5451	-2.112	0.050
2	nNM T vs T— Same FD	nNM T vs T— Different FD	-0.2627	0.5477	-0.480	0.632
2	NM S vs T— Same FD	nNM S vs T— Same FD	-24.492	0.7589	-3.227	0.003
2	NM S vs T— Same FD	nNM S vs T— Different FD	-29.090	0.8198	-3.548	0.002
2	nNM S vs T— Same FD	nNM S vs T— Different FD	-0.4598	0.5008	-0.918	0.441
2	NM S vs FG— Same FD	NM S vs S— Same FD	0.2959	0.4502	0.657	0.584
2	NM S vs FG— Same FD	NM T vs T— Same FD	0.2696	0.4506	0.598	0.586
2	NM S vs FG— Same FD	NM S vs T— Same FD	18.124	0.7737	2.342	0.034
2	NM S vs FG— Same FD	NM T vs FG— Same FD	-10.381	0.3683	-2.819	0.010

FG: fungus garden ants; S: sucrose-treated ants; T: tetracycline-treated ants; FD: faecal droplets; NM: nestmate; nNM: non-nestmate. Significant results are in bold.

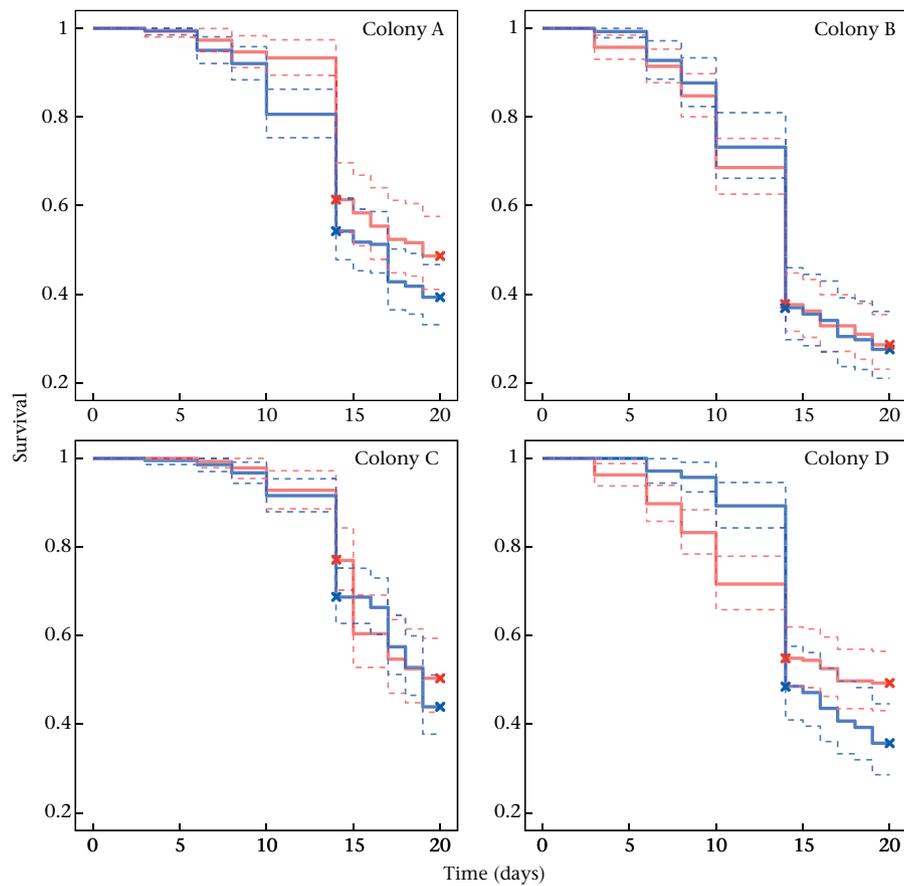


Figure A1. Colony-specific survival curves of ants kept on artificial diets. For each colony, two survival curves are presented: one for ants reared on sucrose diet in Round 1 and then on sucrose + faecal droplets in Round 2 (red) and one for ants reared on tetracycline diet in Round 1 and on sucrose + faecal droplets in Round 2 (blue). Crosses indicate censored data (days 14 and 20). Dashed lines represent the confidence intervals for each survival curve.

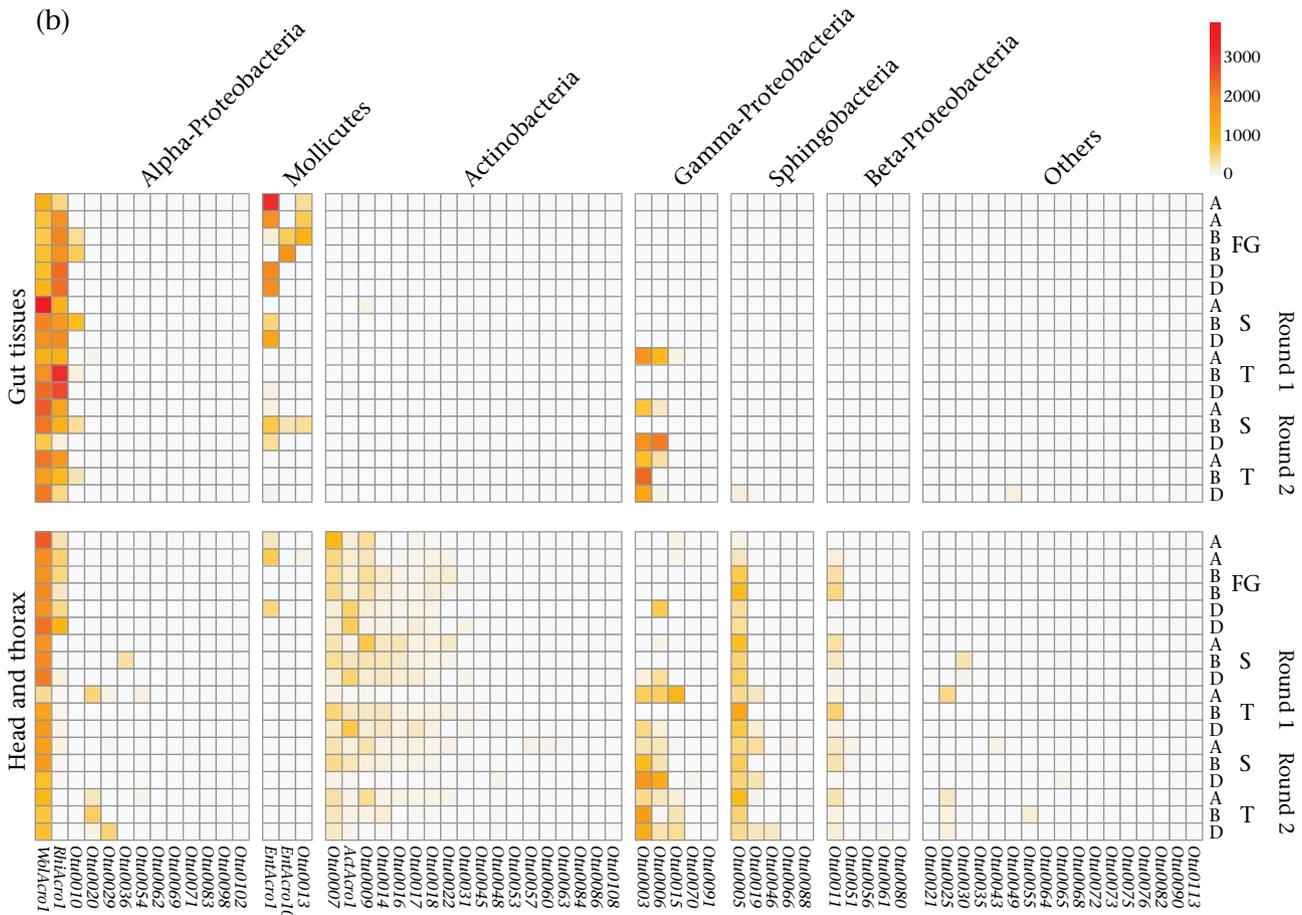
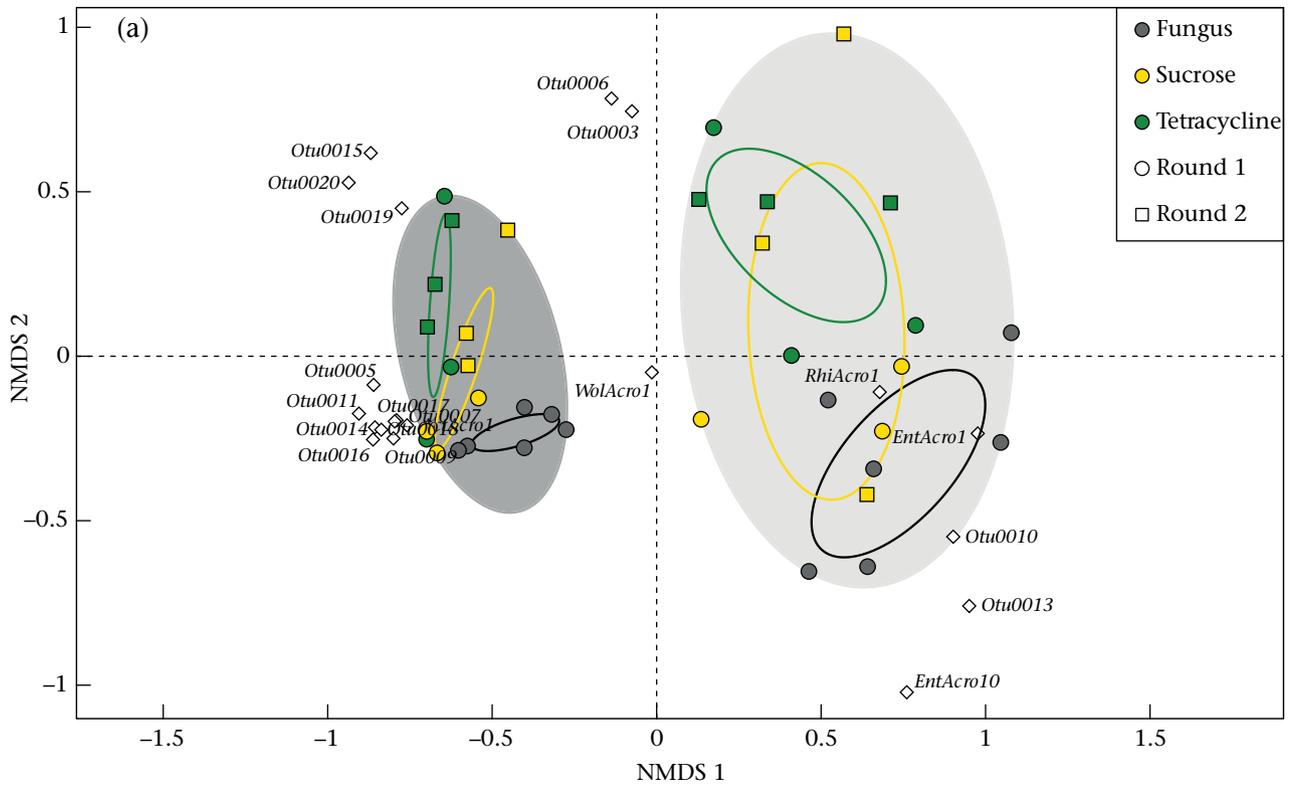


Figure A2. Effects of diet on bacteria in the guts and other tissues (heads and thoraxes). (a). Nonmetric dimensional scaling (NMDS) of Bray–Curtis distances using the rarefied 16SMiSeq reads of the 20 most abundant bacterial OTUs (OTUs with ≥ 1000 reads in total abundance) in both guts and the other body tissues (diamond shapes). Light grey area:



Figure A3. Behaviours observed in the aggression trials. (a) Avoidance, (b) biting, (c) antennation and (d) mandible opening.

clustering of the gut samples; dark grey area: clustering of the head and thorax tissue samples. Coloured ellipses cluster samples based on treatment while symbols show the round of treatment (top right legend). (b) Heat map showing the relative abundances of the most abundant 66 of 411 OTUs after rarefaction (none of the remaining OTUs had more than 400 reads in total and therefore appeared as white blocks when plotted on a heat map). OTUs with ≥ 20 reads in total abundance were grouped by class: the first five belong to Alpha-Proteobacteria, the next three to Mollicutes, then Actinobacteria, Gamma-Proteobacteria, Sphingobacteria, Beta-Proteobacteria and others. The top half of the heat map shows relative abundances of OTUs in gut tissues, while the bottom half shows relative abundances of OTUs in the remaining tissues (heads and thoraxes). The diet treatments from which the ant samples originate are shown towards the right together with colonies of origin (A, B, D) and the round of treatment (1 or 2), similar to the main experiment (see Fig. 1). Detailed information on OTUs is provided in data set S2. FG = fungus garden, T = tetracycline-treated, S = sucrose-treated.



Review

An Overview of the Elusive Passenger in the Gastrointestinal Tract of Cattle: The Shiga Toxin Producing *Escherichia coli*

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Abstract: For approximately 10,000 years, cattle have been our major source of meat and dairy. However, cattle are also a major reservoir for dangerous foodborne pathogens that belong to the Shiga toxin-producing *Escherichia coli* (STEC) group. Even though STEC infections in humans are rare, they are often lethal, as treatment options are limited. In cattle, STEC infections are typically asymptomatic and STEC is able to survive and persist in the cattle GIT by escaping the immune defenses of the host. Interactions with members of the native gut microbiota can favor or inhibit its persistence in cattle, but research in this direction is still in its infancy. Diet, temperature and season but also industrialized animal husbandry practices have a profound effect on STEC prevalence and the native gut microbiota composition. Thus, exploring the native cattle gut microbiota in depth, its interactions with STEC and the factors that affect them could offer viable solutions against STEC carriage in cattle.

Keywords: cattle; STEC colonization; microbiota; bacterial interactions

1. Introduction

The domestication of cattle, approximately 10,000 years ago [1], brought a stable supply of protein to the human diet, which was instrumental for the building of our societies. As the earth's population continued to grow, nutritional demands increased, pushing us towards industrial farming approaches, which can increase meat and dairy production. These approaches include the use of high energy feeds (e.g., starchy grain) [2]; the use of distillery waste products as feed [3]; the use of growth-promoting antimicrobials in several non-EU countries [4]; and the use of crowded industrialized farms [5]. The use of feedlots, which is a common practice in the US and Canada, even though it increases the density of animals per square meter, favors the spread of zoonotic foodborne pathogens [5]. High energy feeds, administered to the animals in order to increase weight gain, also increase the risk of meat and dairy contamination with foodborne bacterial pathogens [6].

One of the most dangerous foodborne pathogens that is occasionally present in cattle GIT is the enterohemorrhagic *Escherichia coli* serotype O157:H7. The serotype O157:H7 belongs to the enterohemorrhagic *Escherichia coli* (EHEC) group and is part of the bigger group of Shiga toxin-producing *Escherichia coli* (STEC). EHEC serotypes are typically defined by their ability to cause disease in humans, hence all STEC are potential pathogens, but unless they have demonstrated pathogenicity to human hosts, they are not classified as EHEC [7–10]. EHEC serotypes O157:H7, O26:H11, O145:H28, O103:H2, O111:H8 and others pose a significant threat to human health [9,11] and their presence in food products has resulted in epidemics in Europe, the US and Japan [12–14]. The serotype O157:H7 alone is estimated to be responsible for approximately 405 million USD loss every year [15]. Cattle are the primary

reservoir of STEC and healthy asymptomatic carriers that can occasionally excrete STEC in their feces. Animals that excrete $>10^4$ STEC CFU/g feces are known as “super-shedders” [16,17]. Strategies to limit STEC in dairy farms include vaccination, bacteriophages, feed additives and direct-fed microbials (DFMs) [18,19]. DFMs offer a promising alternative, as they have not only been implicated as important agents preventing the colonization of *E. coli* O157:H7 [18,20,21]; they also play important roles in host nutrition, as they can protect against ruminal acidosis and they have beneficial immunomodulatory effects [22,23]. However, only a few DFMs have demonstrated efficiency in reducing the shedding of STEC O157 under commercial farm conditions [21] and new candidates are needed.

This review aims to gather existing knowledge in the areas of STEC carriage in bovine hosts and the strategies to control it, but also to highlight the potential of the native microbial symbionts of cattle to prevent zoonotic STEC from colonizing the gastrointestinal tract (GIT). Thus, we present literature on STEC carriage in bovine hosts, but also relevant information on members of the native GIT bacterial community, bacteriophages and ciliate protozoa, which are the groups that offer the greatest potential for inhibiting STEC. We discuss the factors that affect the composition of microbial symbionts and STEC prevalence and present the methods that are currently used to prevent STEC carriage. By comparing STEC and GIT microbiota and discussing the underlying mechanisms that affect their prevalence and abundance, we attempt to highlight the entangled, often hidden relationships between STEC and microbiota, which we could potentially use to prevent STEC carriage.

2. STEC: The Elusive GIT Passenger

2.1. STEC Definition and Pathology

The common pathogenic *E. coli* strains that can cause enteric disease in humans, are classified in seven pathotypes: the enteropathogenic *E. coli* (EPEC), the Shiga toxin-producing *E. coli* (STEC), the enterotoxigenic *E. coli* (ETEC), the enteroinvasive *E. coli* (EIEC), the enteroaggregative *E. coli* (EAEC), the diffusely adherent *E. coli* (DAEC) and the adherent invasive *E. coli* (AIEC), although the pathogenicity of AIEC remains controversial [9,24–26]. EHEC causes symptoms that range from mild gastroenteritis to hemorrhagic colitis and hemolytic uremic syndrome (HUS). From a genotypic point of view, EHEC belongs to the larger group of STEC as they all harbor the Shiga toxin gene (*stx*). More than 400 serotypes have been characterized to date and more than half of them have been associated with serious enteric disease [13]. While the virulence level of STEC is considered to be variable, ranging from avirulent to hypervirulent strains [7,8], reports suggest that all STEC have the potential to be pathogenic to humans [9,25,27]. The *E. coli* O157:H7, which is possibly the most well studied serotype, is widely accepted as the etiological agent of many outbreaks: 64% of the HUS cases in Europe between 2002 and 2006 and 60.4% of the cases between 2012 and 2017 were attributed to O157:H7. Other non-O157 strains (e.g., *E. coli* O26:H11, O145:H28, O103:H2, O111:H8) have also been recognized as equally important outbreak agents [9,25].

In the US, O157 and non-O157 STEC strains are estimated to be responsible for more than 175,000 cases annually [25] and are the most common cause of acute renal failure in children [28]. In 2017 in France, 164 cases of children (<15yo) with HUS were detected and the number of cases has almost doubled in the last 20 years [29]. Infections can be caused by only a few cells [30] and even though they can be asymptomatic, in many cases, they are characterized by watery and/or bloody diarrhea, HUS, acute renal failure, microangiopathic hemolytic anemia, and thrombocytopenia [31,32]. The kidneys, the central nervous system, the lungs, the pancreas, and the heart can be affected, with children and the elderly being the most susceptible groups [31]. Various methods have been developed to characterize STEC infections with PCR of STEC virulence genes being possibly the most popular [33]. Even though the number of annual cases is not very high, STEC infections are considered an important public health issue because treatment options are limited: administration of antibiotics is not recommended because it can lead to toxin production and promote the development of HUS [18].

2.2. Virulence Factors

The repertoire of genes encoding virulence factors in STEC strains is largely similar, however, the gene organization can be vastly different on the grounds that they are located in prophages, plasmids, pathogenicity islands (PAIs) and other mobile elements [25]. The locus of enterocyte effacement (LEE) is a PAI that contains many key pathogenic genes such as the necessary components of the Type III secretion system (T3SS), the effector molecules, the translocated intimin receptor (Tir), and the intimin (*eae*). Together, they are responsible for the attaching and effacing (A/E) lesions induced in intestinal epithelial cells [9,25,27,34,35] (Figure 1).

The defining virulence genes (also used as biomarkers) are the *stx* genes: two *stx* genes have been identified, *stx1* and *stx2*, producing four (Stx1a, Stx1c, Stx1d and Stx1e) and twelve subtypes (Stx2a–Stx2l), respectively [9,36]. Out of the two main variants, Stx2 is more diverse and associated with higher pathogenicity [9,25,37]. Stx is only expressed when the phage becomes lytic, during which the STEC host is lysed and the Stx toxins are released [37] (Figure 1). The released toxins can subsequently bind to the globotriaosylceramide receptor (Gb3), which is present in some mammalian cells, and induce cell death. Other virulence agents include the *Nle* effectors [38], which aid in pathogenicity by promoting colonization, inhibiting the host immune system and prolonging infection. Additionally, considering that the most virulent strains have the complete set of *nle* genes, they are likely key virulence factors [25] (Figure 1).

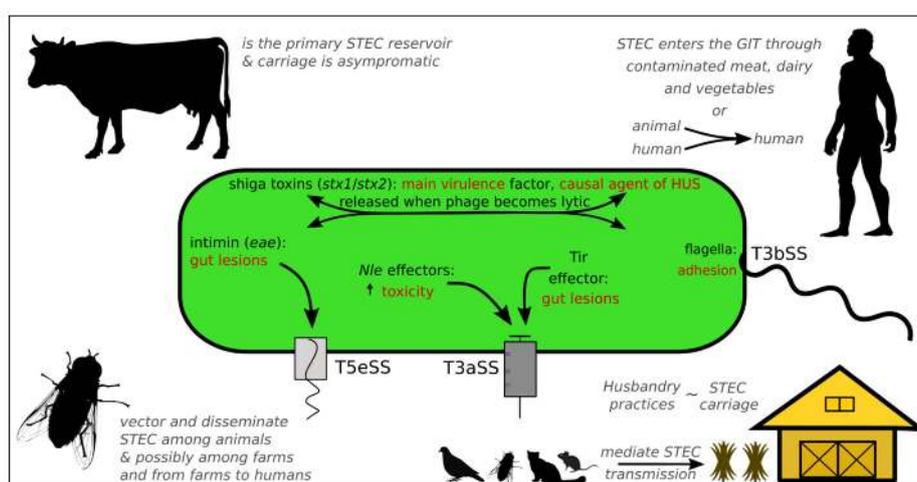


Figure 1. An overview of STEC virulence factors, transmission agents and threat to humans. A STEC cell is illustrated at the center of the figure. Inside the cell, the main virulence factors (black font) and the phenotypes they contribute to (red font) are presented. The primary STEC reservoir is cattle (top left) and contamination occurs through consumption of contaminated meat, vegetables and dairy products and contact with animals carrying STEC or infected humans. *Musca domestica* (the common housefly; bottom left) is a key insect vector for STEC transmission in farms, to vegetables and potentially to humans. Other known STEC vectors include birds, rodents, cats, dogs and ruminants (bottom), but very little research has been conducted in this area [25,39]. STEC levels in cattle vary depending on the season, country and animal husbandry practices (bottom right). Silhouette images were modified from phylopic (phylopic.org), available under a Public Domain License.

2.3. Cattle Are the Main Reservoir of STEC (but in Low Abundance)

Cattle are one of the sources of human STEC infections and many outbreaks have been linked to consumption of undercooked beef or unpasteurized dairy products [12] (Figure 1). Other sources of infection include contaminated vegetables and contact with animals carrying STEC or infected humans [9] (Figure 1). STEC can be present in sheep, wild deer, goats, pigs, pigeons, cats, dogs, rats and rabbits [25], however, cattle are the primary reservoir of STEC O157 and non-O157 serogroups [17,31].

Cattle, like other ruminants (e.g., sheep, goats), are asymptomatic carriers as they lack the vascular receptors (Gb3) for Stx toxins [33]. Therefore, it has been suggested that the aforementioned virulence

factors (Stx, Tir, Eae, Nle) may be relevant for a symbiotic relationship with the adult host [40–43]. Stx can affect functions of T- and B-lymphocytes, suggesting that they could modulate the immune response with possible consequences on STEC survival and colonization of the bovine GIT [44]. STEC immunotolerance in cattle has been attributed to a potential interplay between Shiga toxin and host cells and/or the inability of STEC antigens to reach the immune response sites [45]. It has also been hypothesized that Stx could increase the rate of survival of *E. coli* O157:H7 in the presence of grazing protozoa in the bovine GIT, although such evidence remains contradictory and deserves further investigation [46,47].

STEC abundance is low in cattle; the total amount of *E. coli* cells (which includes STEC) typically accounts for less than 0.01% of the total microbial population in the rumen [48], while in the cecum and feces, *E. coli* accounts for 0.1–1% of the total microbial population [49]. Within farms, the principal mode of *E. coli* O157:H7 transmission among animals is through contact with each other, contaminated feces, feed or water and through houseflies, which are an important *E. coli* O157:H7 vector [19] (Figure 1). STEC can survive in manure and pen floors for periods of up to four months or more [18,50–52], which suggests a high prevalence in the farm environment. Only a few STEC cells are sufficient to colonize a new host [53] and STEC can grow well in open ecosystems [30] and successfully transmit from open environments to new hosts [54]. On the other hand, vertical transmission has not been demonstrated so far, as in an earlier study it was demonstrated that STEC shedding cows had different STEC strains than their calves [55]. Thus, more studies are needed to examine whether STEC can also transmit from mother to offspring, or rather strictly from the environment.

Estimating accurately the prevalence of STEC in cattle is challenging for three reasons: (i) there is no universal method to identify all STEC serotypes in cattle [25,33], (ii) studies have been performed in different conditions, seasons, locations and animals, and (iii) there is a large variability in prevalence. The prevalence can vary from one study to another within a country, and also between countries. A meta-analysis estimated the prevalence of *E. coli* O157 in cattle at the world global level at 5.68%, a higher prevalence being found in US, Australia, Japan and Italy, and the lowest (<1.25%) in Brazil, France, Norway and Germany [56]. Younger animals are more likely to carry *E. coli* O157:H7 [57–59] and non-O157 STEC serotypes [57,60]. This could be related to a less developed immune system, ruminal function and GIT microbiota, as suggested by Zhao [61]. Other potential contributing factors include the preweaning diet and parturition stress, which can lead to increased STEC shedding [60]. Seasonal variation plays an important role as O157:H7 prevalence typically peaks during the summer and the beginning of autumn [12,58,62], while winter conditions seem to favor some non-O157 (i.e., O111 and O145) strains [62]. Seasonal variation is likely related to the temperature and the host physiological response to changing seasons, which further affects STEC transmission, as seasonal changes can trigger enhanced STEC shedding which facilitates rapid spread to new hosts [48]. However, since seasonal variability affects members of the native microbiota as well [63], STEC seasonal variation may also be related to increased/reduced microbial competition. Diets, fasting and farm practices also have a significant effect on the prevalence of STEC in cattle (see below).

2.4. STEC Survival, Colonization and Metabolism in the Bovine GIT

Once ingested by the bovine host, STEC is able to survive, persist and colonize the bovine GIT. *E. coli* O157:H7 transits through the rumen, which is neither a colonization nor a proliferation site as opposed to the intestine (ileum, jejunum, caecum, colon and rectum) [64–66]. Even though not all STEC cells are able to resist the stress induced (acid, low oxygen, osmolytes, low nutrients availability) by the harsh environment of the rumen, some cells are able to persist through a well-regulated response [67,68]. In order to resist the low pH of the abomasum, *E. coli* O157:H7 uses a specialized glutamate dependent acid resistance system, encoded by *gadABC*. Additional resistance systems, such as the Arg system, likely contribute [68], as the complexity of O157:H7 adaptation to low pH is not yet entirely understood [69,70]. Following passage through the rumen, *E. coli* O157:H7 is able to resist the bile [71,72] in the small intestine [73], even though not all STEC are able to do so [71], and reach the rectoanal junction (RAJ) mucosa, which is the principal colonization site [66] (Figure 2).

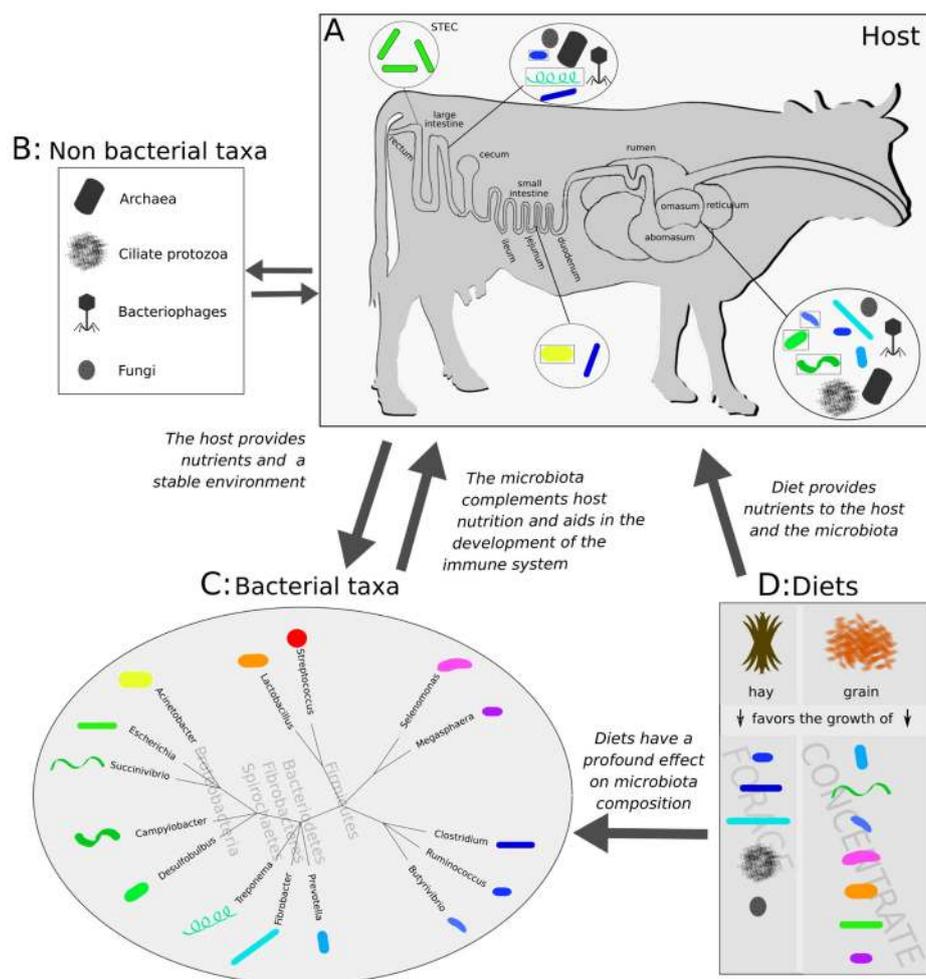


Figure 2. Summary of the most common bacterial and other taxa found in the cattle GIT, their tissue localization and the effect of host diet on their abundance. **A:** A graphic illustration of the cattle gastrointestinal tract compartments. The black circles illustrate the associated microbial communities. Within each circle, the most common genera are presented. Colored illustrations represent bacterial taxa, black and gray illustrations represent microbial taxa and bacteriophages. Black squares around bacterial illustrations indicate that they are part of the mucosa, while bacterial cells with a black outline (top left) indicate the STEC group, which colonizes the RAJ. **B:** Summary of nonbacterial taxa in the GIT. **C:** Bacterial taxa and their taxonomic relationships presented in a phylogenetic tree. The tree is built using the common tree function (www.ncbi.nlm.nih.gov/Taxonomy/CommonTree/wwwcmt.cgi). Phylum names are shown in light gray font behind tree branches. **D:** Association between diet and microbial abundance. Silhouette images were modified from phylopic (phylopic.org), available under a Public Domain License or drawn in Inkscape 0.92.5.

STEC can express numerous molecular determinants involved in surface colonization along the bovine GIT, such as cell surface proteins [74–76]. The T5eSS, Intimin and Tir effector together with the T3aSS EscF-EspABD (which is the molecular structure encoded by the LEE4 operon forming the injectisome) are involved in the formation of A/E lesions localized at *E. coli* O157:H7 microcolonies in RAJ mucosa [77,78] (Figure 1). The injectisome, Tir and Intimin are also required for the colonization of the large and small intestine of cattle as A/E lesions have been observed in the mucosa of the ileum and colon [79,80]. These molecular determinants are globally important surface colonization factors for STEC carriage in ruminants [81–83]. Flagella likely initiate bacterial adhesion to the intestinal epithelium; H7 (flagella) promotes adhesion to rectal epithelium, contrary to H6, H11 and H21 [84], even though both H7 and H6 can bind to bovine mucus (mucins I and II) [85]. Flagella expression (T3bSS) diminishes

after the initial attachment and LEE-encoded components take over [84]. While F9 (fimbriae 9) is clearly involved in the adhesion to rectal epithelial cells [86], the contribution of other pili is likely [87], but hard experimental evidence for the type 1 pili, type 4 HCP (hemorrhagic coli pili), LPF (long polar fimbriae), curli, ECP (*E. coli* common pili), ELF (*E. coli* laminin-binding fimbriae), or SFP (sorbitol fermenting fimbriae) is still lacking [74,88]. Besides multimeric adhesins (injectisome, flagella and pili), the monomeric adhesin EspP (extracellular serine protease plasmid-encoded), which belongs to the T5aSS and is encoded by plasmid pO157, is involved in the adhesion to rectal epithelial cells [77]. Knowledge of the exact role of Intimin in the colonization of the bovine intestine is still lacking, but it could have a similar role to the one that has been described in humans; Intimin exists in 38 distinct subtypes [89] that influence the site of intestinal colonization in humans in a Tir-independent manner, and Intimin appears to restrict colonization in human follicle-associated epithelium [90]. STEC carries additional monomeric adhesins but in-depth investigations are needed to determine their potential involvement in the colonization of the bovine GIT. Some of these adhesins are: the Eha (enterohemorrhagic *E. coli* autotransporters), the Saa (STEC autoagglutination adhesin), the Ag43 (antigen 43), the Sab (STEC autotransporter mediating biofilm formation), the Efa-1 (*E. coli* factor adherence 1), the OmpA (outer membrane protein A) and the Iha (iron-regulated protein A homolog adhesin) [74,76].

3. The Native GIT Microbiota and Its Interactions with STEC

3.1. The Diverse GIT Microbial Community

In order to reach the RAJ, STEC must first transit through the bovine GIT, which is colonized by native microbial symbionts. The rumen is the first GIT compartment STEC enters and is mainly colonized by Bacteroidetes (i.e., Prevotella) and Firmicutes (*Ruminococcaceae*), whose main role is to catabolize complex plant polysaccharides [91–95]. Lactic acid bacteria (LAB; i.e., lactobacilli, streptococci) constitute a smaller part of the rumen community in forage-fed animals [96], but are prominent members in early life and in grain-fed animals [96,97], and they have a demonstrated inhibitory effect against STEC (see below). Ciliate protozoa such as *Epidinium*, *Polyplastron* and *Entodinium* digest structural and storage carbohydrates [98], promote homeostasis by stabilizing the pH in the rumen [99,100], and they can interact with STEC in cooperative and competitive ways (see below). Fungi and archaea are also part of the rumen ecosystem but no studies to our knowledge have suggested an interaction with STEC. Fungi are efficient fiber decomposers and are likely related to xylan and cellulose degradation [98,101,102] and initiation of feed breakdown [101]. Archaea use the hydrogen produced during fermentation to produce methane and prevent H₂ accumulation in the rumen that would inhibit fiber digestion [98,103]. Bacteriophages in the rumen typically belong to the order of Caudovirales [104] and they have been associated with the dominant Firmicutes and Bacteroidetes but also Proteobacteria including STEC [98,101]. The three main roles proposed for the rumen bacteriophages are: (i) the nutrient and enzyme turnover as a result of bacterial cell lysis, (ii) the regulation of dominant species of the bacterial population, and (iii) the horizontal transfer of genes among bacterial species [104].

In the intestine, members of the *Clostridiaceae* family are common in the digesta, while *Acinetobacter*, *Treponema* and *Ruminococcaceae* are rather found in the mucosa, which is also the principal colonization site of STEC [66,105–109] (Figure 2). The epimural microbial communities have been suggested to play a key role in the development and homeostasis of the GIT immune system (especially in early life) [22,98,110]. In addition, they may potentially interact with STEC, since several of these taxa have been identified as over/underrepresented in cattle carrying STEC (Table 1). LAB are also prominent members of the intestinal microbiota as they are dominant in early life calves [111] and appear to protect against gut dysbiosis [110,112]. Both LAB and bacteriophages (of the Caudovirales order) are often isolated from cattle feces and are known to inhibit STEC [113,114] (see below). Other members of the intestinal microbiota include anaerobic fungi and the methanogenic genera *Methanobrevibacter* and *Methanosphaera*, commonly identified in cattle feces [98,105,115], but while their role in the rumen and intestine is often assumed to be similar, there is no experimental evidence whatsoever of their function in the intestine.

Table 1. Summary of 16S comparative metagenomic studies comparing (NS) non-shedders and *E. coli* O157:H7 super-shedders (SS). **A:** Statistically significant differentially abundant taxa between NS and SS (based on the statistical analyses of the cited studies) and the corresponding references are presented. **B:** Additional information about the experimental design of the original studies.

A					
NS			SS		
Phylum	Family/Order	Authors	Phylum	Family/Order	Authors
Actinobacteria	<i>Intrasporangiaceae</i>	Wang et al. [116]	Actinobacteria	<i>Actinomycetaceae</i>	Salaheen et al. [117]
Actinobacteria	<i>Thermomonosporaceae</i>	Salaheen et al. [117]	Actinobacteria	<i>Corynebacteriaceae</i>	Wang et al. [116]
Bacteroidetes	<i>Paraprevotellaceae</i>	Wang et al., Zaheer et al. [116,118]	Actinobacteria	<i>Gordoniaceae</i>	Wang et al. [116]
Bacteroidetes	<i>Prevotellaceae</i>	Zaheer et al. [118]	Actinobacteria	<i>Propionibacteriaceae</i>	Wang et al. [116]
Bacteroidetes	<i>Sphingobacteriaceae</i>	Wang et al. [116]	Actinobacteria	<i>Unclassified Bifidobacteriales</i>	Salaheen et al. [117]
Cyanobacteria	<i>Cyanophyceae</i>	Salaheen et al. [117]	Bacteroidetes	<i>Marinilabiliaceae</i>	Salaheen et al. [117]
Cyanobacteria	<i>Synechococcaceae</i>	Salaheen et al. [117]	Bacteroidetes	<i>Prevotellaceae</i>	Xu et al. [119]
Firmicutes	<i>Bacillaceae</i>	Stenkamp-Strahm et al. [120]	Bacteroidetes	<i>Rikenellaceae</i>	Zaheer et al., Xu et al. [118,119]
Firmicutes	<i>Clostridiaceae</i>	Xu et al., Salaheen et al. [117,119]	Bacteroidetes	<i>Sphingobacteriaceae</i>	Salaheen et al. [117]
Firmicutes	<i>Erysipelotrichaceae</i>	Xu et al. [119]	Firmicutes	<i>Clostridiaceae</i>	Xu et al. [119]
Firmicutes	<i>Heliobacteriaceae</i>	Salaheen et al. [117]	Firmicutes	<i>Erysipelotrichaceae</i>	Wang et al. [116]
Firmicutes	<i>Lachnospiraceae</i>	Wang et al., Xu et al., Salaheen et al. [116,117,119]	Firmicutes	<i>Lachnospiraceae</i>	Stenkamp-Strahm, Xu et al. [119,120]
Firmicutes	<i>Ruminococcaceae</i>	Wang et al., Zaheer et al., Xu et al. [116,118,119]	Firmicutes	<i>Lactobacillaceae</i>	Wang et al. [116]
Firmicutes	<i>Sporolactobacillaceae</i>	Salaheen et al. [117]	Firmicutes	<i>Mogibacteriaceae</i>	Wang et al. [116]
Firmicutes	<i>Streptococcaceae</i>	Salaheen et al. [117]	Firmicutes	<i>Peptococcaceae</i>	Xu et al. [119]
Firmicutes	<i>Veillonellaceae</i>	Wang et al. [116]	Firmicutes	<i>Ruminococcaceae</i>	Zaheer et al., Xu et al. [118,119]
Fusobacteria	<i>Fusobacteriaceae</i>	Wang et al. [116]	Firmicutes	<i>Staphylococcaceae</i>	Wang et al. [116]
Proteobacteria	<i>Alcaligenaceae</i>	Wang et al., Zaheer et al. [116,118]	Firmicutes	<i>Veillonellaceae</i>	Wang et al. [116]
Proteobacteria	<i>Bdellovibrionaceae</i>	Wang et al. [116]	Proteobacteria	<i>Moraxellaceae</i>	Wang et al. [116]
Proteobacteria	<i>Bradyrhizobiaceae</i>	Wang et al. [116]	Proteobacteria	<i>Rhodobacterales</i>	Wang et al. [116]
Proteobacteria	<i>Comamonadaceae</i>	Salaheen et al. [117]			
Proteobacteria	<i>Desulfovibrionaceae</i>	Xu et al. [119]			
Proteobacteria	<i>Nannocystaceae</i>	Salaheen et al. [117]			
Proteobacteria	<i>Rhodospirillaceae</i>	Salaheen et al. [117]			
Proteobacteria	<i>Shewanellaceae</i>	Wang et al. [116]			
Proteobacteria	<i>Succinivibrionaceae</i>	Zaheer et al. [118]			
Spirochaetes	<i>Spirochaetaceae</i>	Zaheer et al. [118]			
Spirochaetes	Unclassified <i>Leptospiraceae</i>	Salaheen et al. [117]			
Spirochaetes	Unclassified Spirochaetales	Salaheen et al. [117]			
Verrucomicrobia	Unclassified Chthoniobacterales	Salaheen et al. [117]			

Table 1. Cont.

B				
doi Number	First Author	Tissue	Country	Animal
10.1128/AEM.01738-17 [116]	Wang	rectum	Canada	beef steers
10.1111/jam.13679 [120]	Stenkamp-Strahm	rectum	US	Holstein-Friesian cows
10.1371/journal.pone.0170050 [118]	Zaheer	lower GI tract	Canada	beef steers
10.1371/journal.pone.0098115 [119]	Xu	rectum	Canada	feedlot steers
10.1016/j.foodcont.2019.03.022 [117]	Salaheen	feces	US	lactating cows

3.2. Interactions between STEC and Native Bacterial Symbionts

In order for *E. coli* O157:H7 to survive and persist in the GIT, it is necessary to either avoid conflict, outcompete or exploit the native microbiota. STEC is able to survive and persist in the GIT through a well-regulated acid resistance system (gadABC). The aforementioned GAD resistance system is activated by the rumen microbiota AHLs signals, which trigger the downregulation of the LEE system and the upregulation of the GAD system. This allows *E. coli* O157:H7 to withstand the low pH in the abomasum [121,122]. *E. coli* O157:H7 relies on the metabolism of galactose, N-acetylglucosamine (GlcNAc), N-acetylgalactosamine (GalNAc), fucose, mannose, and N-acetylneuraminic acid (Neu5Ac). These nutrients are derived from the turnover of enterocytes and the degradation of mucins by mucinolytic symbionts and constitute the carbohydrate portion of the mucus covering the epithelium of the intestine [123]. *E. coli* O157:H7 is able to catabolize in vitro many of these compounds faster than the native microbiota (mannose, GlcNAc, Neu5Ac and galactose preferentially) [123]. Ethanolamine, a carbon and nitrogen source that is abundant in the GIT, as a result of the constant turnover of eukaryotic and bacterial cells, seems to be of particular importance for the colonization of *E. coli* O157:H7. It likely provides a nutritional advantage to STEC because it may not be the preferred substrate for the GIT microbiota [124]. Similar to ethanolamine, aspartate is a key compound for *E. coli* O157:H7 colonization, as mutant assays have demonstrated that it gives *E. coli* O157:H7 a competitive advantage [125]. Similarly, it has been suggested that serine, glycerol and lactate may also be preferred substrates for STEC [126]. The aforementioned nutrients are all present in the GIT [127,128] and although they are not the most abundant nutrients (e.g., GalNAc>fucose: [128]), they are selectively catabolized by *E. coli* O157:H7 [123,125,126,129]. This selective catabolism is possibly part of a niche nutrient specialization that allows *E. coli* O157:H7 to reduce or avoid microbial competition (by catabolizing the less ‘popular’ nutrients) with native GIT endosymbionts. The native GIT microbiota is adapted to the breakdown of complex polysaccharides [94,102], unlike *E. coli* O157:H7, which has a preference for mucus-derived sugars [123,130,131] (a strategy that has also been reported for *Vibrio cholerae* and *Campylobacter jejuni* [123]) and scavenging of other carbon and nitrogen sources (e.g., amino acids, endogenous glycerol [131]). The choice of the nutrients is usually dependent on the growth phase [129,131] and the GIT compartment. Genes encoding proteins involved in the metabolism of ethanolamine are upregulated in STEC O157:H7 in rumen and the small intestine, while those involved in the metabolism of mucus-derived carbohydrates are upregulated in the small intestine and rectum [131]. Nevertheless, the ability of *E. coli* O157:H7 to metabolize these compounds does not always guarantee its survival, as other microbes may be present that can metabolize the same compounds faster or produce antibacterial molecules. For example, *E. coli* O157:H7 can catabolize glycerol [126,131]. *Limosilactobacillus reuteri* (previously known as *Lactobacillus reuteri*; [132]) can also catabolize it and in the process of doing so, it produces HPA that inhibits *E. coli* O157:H7 [20].

3.3. STEC Predation by Phages and Protozoa

Phages targeting *E. coli* O157:H7 have been isolated from humans, animals and other environments where STEC is present [133–136], suggesting a predator–prey relationship. Phages of the *Myoviridae* and *Siphoviridae* families (Caudovirales order) that specifically target *E. coli* O157:H7 have often been isolated from cattle feces [114]. T4-like *Myoviridae* phages have been found in higher titers in low shedding animals, suggesting that their presence is linked to reduced O157:H7 titers [114]. Recent studies have further suggested that cattle intestinal endemic phages target specific STEC strains: *Myoviridae* and *Siphoviridae* phages target various non-O157 groups [137]; *Myoviridae* phages (isolated from cattle feces) target O177, O157, O26 STEC serogroups [138]; a T4-like phage of the *Myoviridae* family isolated from the cattle intestine (not from feces) targeted O157:H7 and non-O157 serotypes but not *Salmonella* strains [139]. Ciliate protozoa have been reported to predate on STEC, but predation does not appear to be STEC specific [47]. In sheep, different species of ciliate protozoa have different interactions, as *Epidinium spp.* appear to have a predatory relationship with STEC, while *Dasytricha spp.* may serve

as hosts to STEC endosymbionts [140]. On the other hand, co-cultures of ciliate protozoa of the genera *Entodinium* and *Epidinium* and STEC showed no interaction between them [141].

4. STEC Emergence in Cattle Farms

4.1. STEC and GIT Symbionts Are Affected by Diet

Dietary change has a profound effect on STEC shedding: distillers grain (DG) has demonstrated a clear effect in increasing O157:H7 shedding [48,142], even though non-O157 STEC serotypes may respond differently [143] and isolated studies have challenged this finding [144]. Barley-feeding and steam-flaked grain (as opposed to dry-rolled grain) have also been associated with increased O157:H7 incidence [48].

Diet also has a profound effect on the GIT microbial communities: unclassified Bacteroidales, unclassified Clostridiales, *Ruminococcaceae* and *Fibrobacter* are more abundant in forage-fed bovines; *Prevotella* and unclassified *Succinivibrionaceae* are enriched in animals fed diets containing concentrate and *Butyrivibrio* in animals fed mixes of forage and concentrate [115] (Figure 2). *Ruminococcus*, one of the dominant bacteria, is evenly distributed between animals fed forage and concentrate, but this is an exception [115]. When animals are fed high grain diets, lactic acid producing (e.g., *S. bovis*, lactobacilli) and lactic acid utilizing taxa (*Megasphaera elsdenii*, *Selenomonas*) increase [22,96]. In contrast, rumen protozoa and anaerobic fungi tend to have lower concentrations in high grain-fed animals, but not always (e.g., cows with diet-induced ruminal acidosis can have a higher abundance of rumen fungi than healthy cows) [98], while the starch-degrading *Entodinium* becomes dominant in high grain diets [101]. Microbiota composition is also affected by feed supplements [145,146], periodical starvation [147] and high fat diets [148].

4.2. Ruminal Acidosis and the Emergence of Acid Resistant Strains

Transition from high-forage to high-concentrate feed can trigger a condition called (sub)clinical acidosis, which is characterized by low ruminal pH (<5.2 or <5.6; the definition varies among studies), increased lipopolysaccharide presence, inflammation and other host-specific negative effects [149]. The condition is commonly distinguished as either a severe (clinical acidosis) or a less severe response (subclinical acidosis), also known as acute or subacute ruminal acidosis (SARA). SARA is often characterized by reversible rumen pH depressions and the production of other toxic biomarkers that can precipitate further health disorders [99]. The effect on microbial communities is more severe in the lumen than the epithelium of the rumen [150,151] and microbial communities in the intestine are affected as well [99]. In the rumen, SARA results in a reduction in microbial diversity and richness that may signify a reduction or shift in functionality and catabolic potential [99]. At the phylum level, a general decrease in Bacteroidetes and Fibrobacteres [150,152,153] and an increase in Firmicutes [99] is observed. An increase in *Prevotella* (both in rumen and rectum) and *S. bovis* (in rumen) has been documented in many studies [150,152–156] and this increase is higher if animals are fed processed DG as opposed to unprocessed grain [106] (Figure 2).

Generic *E. coli* (STEC and non-STEC) has been reported to increase during grain-induced SARA [6,155–157]. On the basis of this finding, it was hypothesized that acidic conditions may promote the emergence of acid resistant STEC [158]. However, the comparison between forage- and grain-fed animals showed that low acidity in the rumen is unrelated to the development of acid resistance, as passage through the rumen is sufficient to turn acid sensitive to acid resistant (AR) STEC [158]. The mechanism that STEC uses to become AR relies upon the activation of the GAD pathway through the AHL compounds presence in the rumen [121]. STEC cannot produce but it can detect AHL signals from different rumen symbionts through the SdiA protein, and upon detection of these signals, the LEE gene expression shuts down, while that of the *gad* genes is upregulated [121]. The SdiA system regulation is functional in both grain and forage diets, even though the GAD activation appears to be more important for the survival of STEC in grain-fed animals [122].

4.3. Other Husbandry Practices Related to STEC Emergence

Fasting, which often occurs during and after the transport of animals, has also been associated with increased pH, decreased SCFA and increased O157:H7 abundance [48]. The administration of antimicrobials, typically added to cattle diet to improve animal growth and weight gain [4], a practice that has been banned in Europe (IP/05/1687), can intensify the *E. coli* O157:H7 threat. Monensin, an antimicrobial commonly used in the US, has no or little effect on gram-negative bacteria (e.g., *E. coli*), and therefore, administration has either no effect on *E. coli* O157:H7 [19,159] or causes an increase in *E. coli* O157:H7 shedding [4]. In studies where monensin administration resulted in a reduction in *E. coli* O157:H7 shedding, the animals received the highest possible concentration (44mg/kg), which possibly resulted in the elimination of beneficial symbionts and reduced fermentation in the hindgut [160].

Animal husbandry practices can further favor *E. coli* O157:H7 transmission in a cumulative manner: when animals in crowded farms are fed high grain, prevalence of *E. coli* O157:H7 increases as super-shedders can transmit O157:H7 to other animals in the same pen [161]. Within farms, feed and water contamination have been suggested to promote *E. coli* O157:H7 transmission. However, fecal contamination of pen floors and animal hides poses an even greater risk for *E. coli* O157:H7 transmission [19]. In a study that examined the potential connection between farm conditions and *E. coli* O157:H7 prevalence, it was demonstrated that muddy pens increase the chance of shedding [162]. Flies that belong to the *Muscidae* and *Calliphoridae* families facilitate *E. coli* O157:H7 transmission to cattle [19] (Figure 1), to vegetation [163] and possibly humans [19]. Finally, animal stress results in the release of norepinephrine in the cattle GIT, which promotes *E. coli* O157:H7 growth [19].

5. STEC Prevention Methods and Future Perspectives

Strategies to control STEC in cattle have so far focused on eliminating STEC in the animal GIT, feces and farm environment (i.e., feed, pen floors). These strategies aim to: (i) limit the occurrence of STEC in meat and dairy products and prevent it from entering the food chain and (ii) reduce the financial losses from products that are discarded as a result of STEC contamination [164]. Most strategies typically aim to reduce the STEC load in cattle, but regular cleaning of feedlots and pen surfaces and provision of dry bedding have also been proposed as methods to reduce STEC prevalence.

5.1. Active and Passive Vaccination

Active (through intramuscular injection) and passive (through colostrum administration) vaccination have been proposed as methods to control STEC. Colostrum administration to newborn calves facilitates the transfer of maternal antibodies (Abs) that protect until the calf develops its own immune responses [110]. Thus, if dams have a significant humoral immune response against STEC, then colostrum administration should facilitate the vertical transmission of STEC-specific Abs, which would result in reduced STEC shedding. However, even though Ab transfer from (vaccinated) dams to their calves through colostrum administration has been demonstrated [165–167], shedding is not always reduced [168]. In addition, results are often hard to interpret [169], as colostrum administration from multiple dams to calves is more efficient in reducing STEC shedding than colostrum administration from their own mother [170]. This would suggest that transmission of maternal Abs may not be the (only) reason colostrum administration reduces STEC shedding, but it may be related to other beneficial effects triggered by colostrum administration, such as the faster or more efficient establishment of GIT microbial symbionts [110].

STEC rarely causes symptoms (except occasional diarrhea in newborn calves) and appears to be immunotolerated in cattle [45,171,172]. It has been proposed by Schaut and colleagues [173] that this could be part of an interplay between STEC and host, similar to the one observed in humans between *E. coli* symbionts and the host. STEC immunotolerance in cattle has also been attributed to low

STEC abundance [171], the inability of Stx to reach the site that initiates an immune response [45] or a potential immunosuppression/immunodelay caused by Stx [172]. On the basis of these previous works, immunization with inactivated forms of Shiga toxin was suggested and trials have demonstrated somewhat promising results [173,174]. Active vaccination targeting STEC Type III secreted proteins (e.g., Esps, Tir) has demonstrated a reduction in *E. coli* O157:H7 shedding [175] although the effect is not consistent [176] and varies among animals. Therefore, even though vaccination holds significant theoretical promise that has led to field trials and commercialization of anti-EHEC vaccines (Canada), the tests were not sufficiently encouraging to result in widespread use [177].

5.2. Bacteriophages

The use of bacteriophages, typically isolated from cattle feces, against *E. coli* O157:H7 and non-O157 has reported promising results. However, the evaluation of their efficiency can be challenging, as the presence of other phages in the cattle GIT can make interpretation of results difficult [178]. STEC control using bacteriophages against STEC contaminated surfaces in cattle farms (e.g., cow hides) and meat products has demonstrated a significant decrease in STEC [114,139]. However, even though in vitro assays have shown promising results [137–139], in vivo trials have not always been successful. Administration of bacteriophages can result in STEC reduction, although in some studies, the reduction between treatment groups was not significant and in some other studies, there was no reduction at all [114].

5.3. Non-Microbial Feed Additives

Various feed and water additives, such as orange, citrus peel and pulp and tannin extracts, have reported positive results in controlling *E. coli* O157:H7 in cattle [48]. Lactoferrin, an iron-binding glycoprotein that exhibits antibacterial activity, can eliminate *E. coli* O157:H7 shedding if administered by rectal injection but not orally [179]. However, most of these results originate from small trials and single studies, and therefore, extensive testing is required to verify their potential anti-O157:H7 properties and examine their impact on the rest of the microbiota (since many antimicrobials are wide range) [19,48].

5.4. Direct-Fed Microbials

DFMs can effectively reduce the *E. coli* O157 shedding in cattle [21] and the summarized beneficial modes of action that are likely responsible are: (i) competitive exclusion, (ii) production of antimicrobial compounds, (iii) reduced risk for ruminal acidosis [99] and (iv) potential beneficial interactions with members of the GIT microbiota. LAB strains isolated from cattle feces have shown promising results against *E. coli* O157 through lactic acid production and pH reduction [113,158]. The administration of combined *Lactobacillus acidophilus* and *Propionibacterium freudenreichii* against *E. coli* O157 shedding has been widely tested and showed better efficacy than other DFMs [18,21]. Combined *Streptococcus bovis* and *Lactobacillus gallinarum* have also reported promising results [21,180]. In one of the first studies that used DFMs against *E. coli* O157:H7, the reduced shedding was attributed to an increase in SCFA in feces, which was suggested to have bactericidal (anti-gram negative) activity [180]. In a recent study, Bertin and colleagues [20] demonstrated that *L. reuteri* catabolizes glycerol to produce HPA, which inhibits *E. coli* O157:H7 in vitro. Administration of DFMs also has an important role in early life cattle; if *Faecalibacterium prausnitzii* is administered early in life, it can reduce the incidence of diarrhea (caused by *E. coli* pathotypes) as it persists within the digestive tract for several weeks [110].

5.5. Towards New Strategies for STEC Control

Considering the effectiveness of DFMs against STEC colonization in cattle, the rich diversity of cattle GIT microbiota and the low abundance of STEC in the GIT, it is very likely that the GIT microbiota offers a promising field for the discovery of next generation DFMs. Several lines of indirect evidence support this hypothesis; even though STEC is very prevalent in the cattle farm environment [50–52] and is easily transmitted among animal hosts [53,54], its prevalence and abundance in cattle is never high

(see above). This suggests that there are host or other factors preventing its colonization. Since healthy cattle carry STEC asymptotically and STEC carriage does not affect cattle growth or reproduction, natural (host) selection for defenses against STEC colonization is “invisible”. However, natural selection against STEC could be triggered by conflict with the native GIT microbiota, possibly a result of competition between gut symbionts and STEC for resources.

The existence of STEC antagonists within the cattle microbiome has further been suggested by 16S metagenomic studies that compared the rectal microbiota of super-shedders (SS) and non-shedders (NS) in order to identify potential patterns of mutually exclusivity or co-occurrence between *E. coli* O157:H7 and members of the microbiota [116–120]. These studies took place explicitly in the US and Canada and mainly focused on steers fed diets that included grain (Table 1). Even though comparisons of rectal bacterial communities in SS and NS did not demonstrate sharp contrasts in α - or β -diversity, all studies identified some taxa (at phylum, family or genus level) with a preference towards SS or NS. Members of Firmicutes (order Clostridiales) and Bacteroidetes (order Bacteroidales), which are also the most prevalent phyla in the cattle GIT (Figure 2), are the most commonly differentially represented taxa in SS and NS animals (Table 1). Bacterial taxa belonging to *Ruminococcaceae*, *Paraprevotellaceae* or *Alcaligenaceae* are present more in NS. Taxa within *Prevotellaceae*, *Clostridiaceae*, *Erysipelotrichaceae*, *Lachnospiraceae* and *Ruminococcaceae* appear overrepresented in both NS and SS (Table 1). Although pioneering, these correlative studies need to be followed up by shotgun metagenomics/whole genome sequencing and comparative genomics, which will allow us to better identify potential STEC antagonists and examine their genomic potential. Such insights can then be followed up (whenever possible) by in vitro experiments. The search for STEC antagonists in the cattle microbiota has the potential to identify specialized gut symbionts that have adapted to an endosymbiotic lifestyle and possibly coevolved with the cattle host. Such symbionts are likely to provide mutualistic services to the host (nutritional and other)—in addition to preventing STEC colonization—and therefore, this type of research could identify DFMs that will not only eliminate STEC but may also improve the wellbeing and productivity of ruminants.

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