



Contribution à l'analyse des interactions tripartites entre *Brassica napus*, *Delia radicum* et leur microbiote

Morgane Ourry

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COMUE UNIVERSITE BRETAGNE LOIRE

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Par

Morgane OURRY

**Contribution to the analysis of tripartite interactions between
Brassica napus, *Delia radicum* and their microbiota**

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Isaac Newton*

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“L’arrivée des premiers extraterrestres sur Terre passa inaperçue aux yeux des humains. Leur espèce était microscopique, et ils étaient venus saluer les Tardigrades, qu’ils considéraient comme les véritables maîtres de la planète.” Nanofictions – Patrick BAUD



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*Mersi bras deoc'h tout, kalon vat ha ken
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ABBREVIATIONS LIST

A

AAs: Amino Acids
ANOVA: ANalysis Of Variance
ASVs: Amplicon Sequence Variants

B

BSA: Bovine Serum Albumin
Bt: *Bacillus thuringiensis*

C

CFU: Colony Forming Unit
CLM: Cumulative Link Model
CPOs: Carbohydrate, Polyol and Organic Acids
CRF: Cabbage Root Fly

D

DADA: Divisive Amplicon Denoising Algorithm
DAI: days after infestation
dbRDA: distance-based redundancy discriminant analysis
DNA: DeoxyriboNucleic Acid
DIABLO: Data Integration Analysis for Biomarker discovery using Latent cOmponents

E

EcoFAB: Ecosystems FABrication

F

FDR: False Discovery Rate
FISH: Fluorescence In Situ Hybridization

G

GC: Gas Chromatography
GFPs: Green Fluorescent Proteins
GLM: Generalized Linear Model
GSLs: Glucosinolates
gyrB: DNA gyrase subunit B

H

HIPV: Herbivore-Induced Plant Volatile

J

JA: Jasmonic Acid

L

LHTs: Life History Traits
LM: Linear Model

M

MW: Molecular Weight

O

OSR: Oilseed Rape
OTUs: Operational Taxonomic Units

P

PCoA: Principal Coordinate Analysis
PCR: Polymerase Chain Reaction
PGPR: Plant Growth Promoting Rhizobacteria
PICRUST: Phylogenetic Investigation of Communities by Reconstruction of Unobserved States

R

RDA: Redundancy Analysis

S

SA: Salicylic Acid
SMCSO: S-Methyl Cysteine Sulfoxide
SOAs: sugars and organic acids
SynComs: Synthetic Communities

T

tb-RDA: transformation-based redundancy analysis
TSA: Tryptic Soy Agar

U

UPLC: Ultra Performance Liquid Chromatography

V

VOCs: Volatile Organic Compounds

FOREWORD

The notion of “symbiosis” was first used in the mid-19th century by Anton de Bary and can be referred to as “the living together of unlike named organisms” (Sapp, 2010). This term encompasses a large range of interactions but is frequently used to describe mutualistic interactions involving symbionts, and many studies highlighted the great impact that symbionts can have on their host. In 1993, Lynn Margulis wrote that “all life on Earth forms one complex interacting system”, introducing the concept of holobiont where a macroorganism (i.e. a host) and its microorganisms (i.e. its symbionts) form one complex biological unit.

Plant-insect interactions are often studied to understand defensive mechanisms and how they are implemented by the plant when attacked by herbivorous insects (Fürstenberg-Hägg *et al.*, 2013; Aljbory and Chen, 2018). Both plants and insects harbor symbionts and therefore can be considered as holobionts (Douglas, 2015; Vandenkoornhuyse *et al.*, 2015). In the former, symbionts can promote plant growth and enhance its defenses against insects whereas in the latter, symbionts can improve the insect fitness and help them to cope with plant defenses. While these two research topics were mostly studied with microorganisms at the species scale, recognition of the microbial community scale importance within plant-insect-microorganisms interactions is arising.

The present work has targeted this point as we studied tripartite interactions between a plant, an insect and their associated microbial communities. This thesis was divided into three objectives: i) the first objective was to determine the effect of soil microorganisms on plant chemistry and insect life history traits; ii) the second objective was to assess how the insect root herbivory influences back the plant root chemistry and microbial communities throughout the insect development and to identify potential relationships between chemical and microbial modifications following herbivory; iii) the third and last objective was to evaluate insect bacterial communities at the adult stage and to investigate insect bacterial transmission pathways. We focused on these three objectives using a crop plant, the winter oilseed rape (*Brassica napus*) and a root herbivore, the cabbage root fly (*Delia radicum*).

This thesis is the successful result of collaborations between the Resistance and Adaptation team and the Ecology and Genetics of Insects team, at the Institute for Genetics, Environment and Plant Protection (UMR 1349 INRA, Agrocampus Ouest, Université de Rennes). It was co-funded by a grant from the Plant Health and Environment division of the French National Institute for Agricultural Research (INRA) and the French Brittany Region.

CHAPTER I

GENERAL INTRODUCTION



CHAPTER I: GENERAL INTRODUCTION

1. Conceptual framework

1.1. Symbiosis concept

1.1.1. Definition and actors

The notion of “symbiosis” was first used in the mid-19th century by Anton de Bary when mentioning the fungi-algae association, also called lichen (Zilber-Rosenberg and Rosenberg, 2008). Symbiosis was a neutral term to name “the living together of unlike named organisms”. It usually concerned two living organisms interacting with each other, but this term was then frequently associated to mutualistic interactions (Sapp, 2010). While many researchers debate about the definition of symbiosis, biotic interactions were classified according to their nature, having either a positive, neutral or negative effect on the fitness of the interacting organisms, from mutualism to competition as displayed in figure 1.

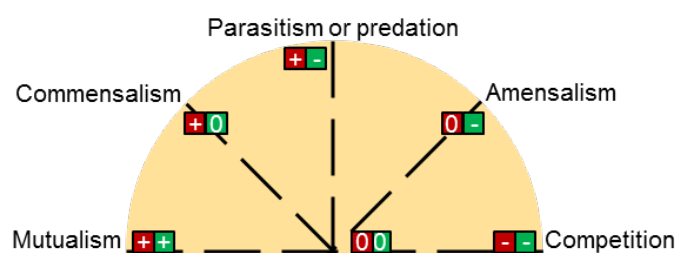


Figure 1 Possible biotic interactions that can occur between two living organisms. These two organisms are respectively represented by green and red colors. A “+” symbol represents a positive effect of the interaction on the fitness of an organism while “-” and “0” symbols represents respectively a negative and no effect.

Various organisms can be involved in symbiotic interactions as they can be: of different kingdoms (Ruggiero *et al.*, 2015) like ants and acacia trees protecting each other and of different size (McFall-Ngai, 2014) like squid acquiring bioluminescence from the bacteria *Vibrio fischeri*. Such interactions can occur at different scales. They can be internal where bacteria such as *Buchnera* live inside aphid cells (i.e. bacteriocytes) and are essential for the insect development (Douglas, 1998) or external, like root nodules formed by the symbiosis between legume roots and nitrogen-fixing bacteria (Richardson *et al.*, 2009). They can also occur in different ecosystems, terrestrial environment where ants breed aphids (Offenberg, 2001) or marine environment where sea anemones protect clown fishes (Feeney and Brooker, 2017).

In this manuscript, symbiosis will be mostly used to talk about beneficial interactions between macro- and microorganisms.

1.1.2. Symbiosis consequences

Symbiotic interactions between different organisms can lead to various changes for one or both organisms at different levels: morphological, physiological or behavioral, but changes in genetics and evolution processes can also occur.

A frequently occurring morphological modification would be mycorrhizae, present in most plants on the planet. Mycorrhizae result from plant roots and fungi symbiosis where hyphae will surround or penetrate the plant cells within the root cortex, hence extending the plant root architecture and capacity to acquire phosphorus, hence improving the plant nutrition, growth and health (Richardson *et al.*, 2009).

The previous case is also an example of physiological modifications where mycorrhizae improve the plant phosphorus nutrition, while root nodules enhance nitrogen nutrition of plants. Insect nutrition can also be improved by microbial symbionts as it is the case for aphids where *Buchnera* provides essential amino acids to its host (Douglas, 1998). This bacterium has become an obligate symbiont since it is essential to aphids for a successful development. Reproduction function can also be modified following a symbiotic interaction like with the intracellular bacterium *Wolbachia*, found in arthropods and nematodes, and which is able to induce four different reproductive phenotypes (feminization, parthenogenesis, male killing, cytoplasmic incompatibility) that change both the reproduction and the offspring (Werren *et al.*, 2008).

Behavioral modifications are quite known in the case of parasitism. Such a change can also affect survival rate especially when a parasitic interaction leads to the host “suicide” so the parasite can continue its cycle in another host. Gordian worms manipulate their grasshopper or cricket host so it jumps in water while another worm, lancet liver fluke (*Dicrocoelium dendriticum*), makes its ant host to be eaten by grazing mammals (Libersat *et al.*, 2009).

The discovery of such interactions have led a new concept: the extended phenotype developed by Dawkins (1982). He stated that “an animal's behavior tends to maximize the survival of the genes “for” that behavior, whether or not those genes happen to be in the body of the particular animal performing the behavior”. To simplify, an animal behavior that can be observed is not necessary caused by the animal genes but of others. Parasitism illustrates well this concept and Hughes (2013) considered three cases of extended phenotype: animal

architecture as a physical representation of the animal behavior, parasite manipulation of host behavior and action at distance of host behavior manipulation. In addition to morphological and behavioral changes, Schaedelin and Taborsky (2009) broadened the concept by accepting signals (e.g. chemical compounds produced by an organism) like a manifestation of an extended phenotype.

1.2. The holobiont concept

1.2.1. Definition and actors

The concept of symbiosis has been broadened over the last decades and Lynn Margulis wrote that “all life on Earth forms one complex interacting system” (Margulis, 1993). This statement was the precursor of a new concept: the holobiont, where the prefix “holo” from the Greek ὅλος (*hólos*) means whole while βίος (*bíos*) means life. For example, this term implies that a macroorganism (i.e. a host) and its microorganisms (i.e. its symbionts) form one complex biological unit, or a “superorganism” according to some researchers, even though the latter term should be used carefully according to the situation. Bordenstein and Theis (2015) tried to establish a conceptual framework using ten principles to describe the notion of holobiont and that could serve as a roadmap for hypothesis-driven research on holobionts. In this paper, the authors described holobionts as units of biological organizations using more accurate and appropriate vocabulary while taking into account the notions of variations associated to genetics and mutations, of evolution according to Lamarck and Mendel, of natural selection and speciation. Underlying the concept of holobiont are the notion of coadaptation and coevolution. Indeed, displaying a mutualistic interaction between a host and microorganisms implies that both parties must have either implement adaptation (e.g. genes or phenotypic traits) in response to the same selective pressure, or evolve by exerting selective pressure on each other.

The concept of the holobiont is now frequently used when studying an organism being either an invertebrate or a vertebrate (Rosenberg and Zilber-Rosenberg, 2018) and biological models such as human (Cryan and Dinan, 2012), animals like insect (Douglas, 2015) or plants (Vandenkoornhuyse *et al.*, 2015) are known to host plenty of symbionts. These symbionts can be more largely called “microbiota” that consists of the assembly of microorganisms (community) belonging to different kingdoms (Prokaryotes [Bacteria, Archaea], micro-Eukaryotes [e.g. Protozoa, Fungi, Algae]) in a defined environment which interact together. Indeed, these microorganisms can either be mutualistic, commensal, pathogenic or competitive depending on the other interacting party (figure 1). The notion of “microbiome” can correspond

not only to the microbiota, but also to their functions and activities (i.e. structural elements like genomes and metabolites) as well as the surrounding environmental conditions.

1.2.2. Emergence of the hologenome concept

As any “organism”, a holobiont is also associated to a genome, and more precisely to a “hologenome” (Zilber-Rosenberg and Rosenberg, 2008; Rosenberg and Zilber-Rosenberg, 2018). The hologenome of a holobiont corresponds to a genetic unit, a combination of the host genome and the metagenome of its associated communities, which is the microbiome (Bordenstein and Theis, 2015).

However, this concept is controversial and source of many debates especially when talking about the “hologenome theory of evolution”, first developed by Zilber-Rosenberg and Rosenberg (2008). Some researchers stated that seeing the hologenome as a unit of selection is an oversimplified view since most microorganisms that interact with plants or animals are non heritable (Moran and Sloan, 2015; Douglas and Werren, 2016). Another debate is about whether host and microorganisms evolve together as a unit (Moran and Sloan, 2015). Indeed, an intimate relationship does not necessarily mean that there is a history of coevolution between the host and its microorganisms and if there is coevolution, it does not mean that these species evolve in response to each other. While closely related hosts have higher chances to share similar microbial communities than distantly related hosts, it is not possible to determine whether it is because of coevolution or shared evolutionary history. Moran and Sloan (2015) concluded that we should not assume that “associated organisms evolve to function as a cooperative unit” and that empirical studies are necessary to understand the evolution of host-microorganism interactions.

1.2.3. Holobionts as dynamic units: sources of dynamics

Plants and animals are living in interaction with their associated microbial communities, which are vertically transmitted (i.e. from the parents to their offspring) and/or can be acquired through horizontal transfers (i.e. from the environment or other organisms). Moreover, it is largely admitted that spatiotemporal factors affect such host-microorganism interactions. For example, it has been demonstrated that bacterial and fungal communities vary through the plant development (Mougel *et al.*, 2006; Hannula *et al.*, 2010; Chaparro *et al.*, 2014). The same goes for insects, which development stages affect microbiota, especially when a larva undergoes metamorphosis and that its diet changes drastically, hence modifying its gut microbiota (Paniagua Voirol *et al.*, 2018). Moreover, plant microbiota changes according to the

environment the plant grows in: in different soils (Dombrowski *et al.*, 2017), in different agricultural practice systems (Hartmann *et al.*, 2015), at different seasons (Agler *et al.*, 2016). Biotic factors such as pathogens (Lebreton *et al.*, 2019) or insects (Yang *et al.*, 2011) also play a role in shaping microbial communities.

In this manuscript, plants and insects will be considered as holobionts, thus as dynamic units, which microbial communities could possibly change over time and location and depending on the biological interactions that are occurring.

2. Plant-insect interactions within the “holobiont” concept – how and to which extent can these interactions be modulated by microorganisms?

2.1. General context

Herbivory, also called phytophagy, is the act performed by animals feeding on plant tissues (i.e. leaf, stem, root) or products (i.e. flowers, fruits), and we differentiate aboveground from belowground herbivory. In the following manuscript, we will talk about herbivorous or phytophagous insects, or more concisely herbivores and phytophages.

Among herbivorous insects, there are phloem-feeding or piercing-sucking insects, which possess a tube-like structure they insert in the plant cell to suck the liquid inside (i.e. the sap from the phloem) and grazing or chewing insects, which have developed specialized mouthparts for chewing and tearing the plant tissues. They can also be classified according to their specificity, feeding guild or diet, by being either generalist where insects are polyphagous as they have a large host range, or specialist where they have a narrower host range as they can develop only on one or few plant species from the same family (i.e. mono- or oligophagous respectively). In the latter case, insects must be well adapted to resist or counter plant defenses compared to generalists. While a generalist performance is usually lower than a specialist on the same host plant species (Arany *et al.*, 2008), plants also react differently to herbivores depending on whether it is a generalist or a specialist insect. For example, the production of floral volatiles can vary according to the presence of specialist or generalist aphids (Pareja *et al.*, 2012).

2.2. Plant reactions to herbivory: a display of controlled defenses

To defend themselves, plants use different strategies: constitutive defenses, which chemical compounds and physical barriers are produced or maintained at all times by the plants, and induced defenses, which are synthesized following a wound or an attack from a herbivorous insect. In the latter case, elicitors like insect oral secretions, regurgitate food, feces or honeydews are able to activate the productions of plant direct and indirect defenses (Howe and Jander, 2008; Erb *et al.*, 2012). Figure 2 summarizes the plant defenses described in the present section.

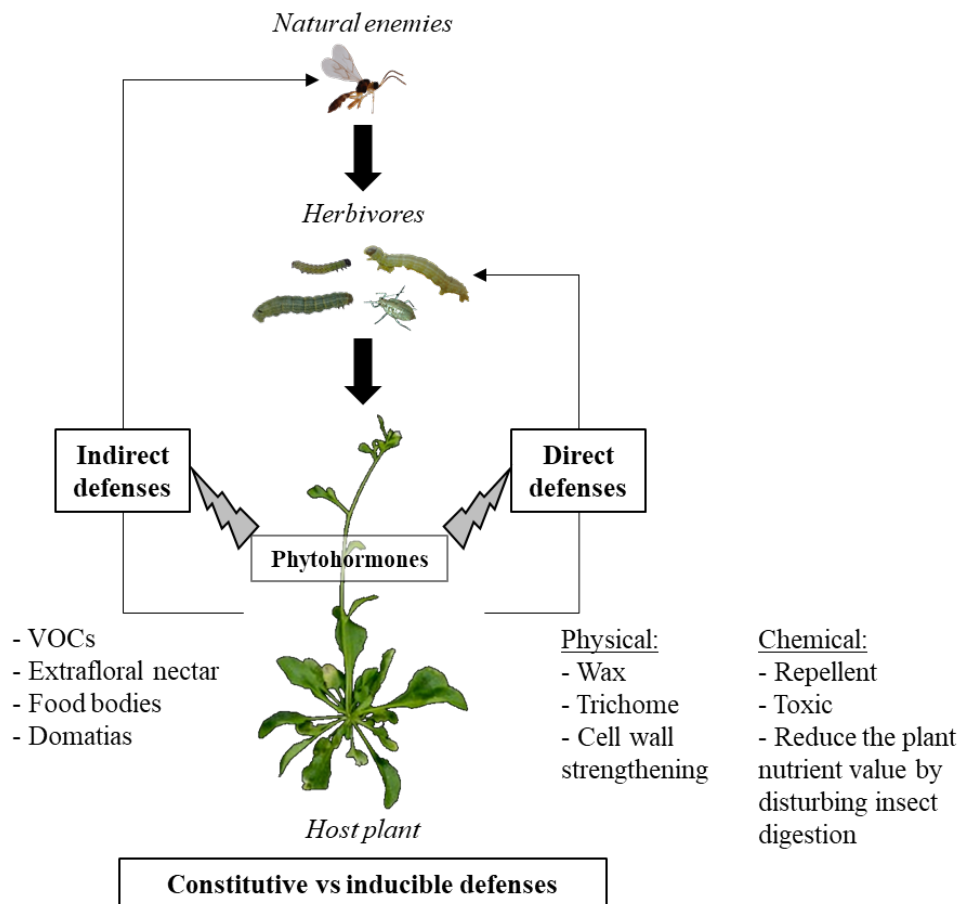


Figure 2 Overview of the plant indirect and direct defenses against herbivorous insects.

2.2.1. Direct defenses

Direct defenses correspond to constitutive and inducible, either physical or chemical, defensive mechanisms that counter herbivorous insects (Chen, 2008; Fürstenberg-Hägg *et al.*, 2013).

Physical defenses are associated to plant morphological features that will prevent herbivory. For example, plants can display different types of physical barriers, which limit the food supply for herbivorous insects, like wax-type films increasing the leaf slipperiness, fine outgrowths (i.e. trichomes), or cell wall strengthening via an accumulation of lignine or cellulose in the plant tissue (Fürstenberg-Hägg *et al.*, 2013).

Chemical defenses correspond to the production or the increase of plant chemical compounds following herbivory, and that differently affect herbivores depending on the compound or insect characteristics. While primary metabolites, like amino acids and sugars, intervene in the plant primary functions (i.e. growth, development and reproduction), secondary metabolites are associated with secondary functions like the plant protection. This includes the establishment of defenses against herbivorous insects that can be non-toxic, toxic and/or

repellent. Non-toxic secondary metabolites, like for example proteinase inhibitors, have the capacity to disturb the herbivore digestion. These enzymes inhibit insect digestive enzymes, which results into lowering the availability of amino acids, hence reducing the plant nutrient values (Fan and Guo-Jiang, 2005; Fürstenberg-Hägg *et al.*, 2013). Toxic or repellent secondary metabolites are a large group of compounds belonging to different chemical families, with the main ones being: alkaloids, terpenoids, flavonoids, glycosides and phenols. We will be focusing on the glycosides, and more specifically on glucosinolates (GSLs).

GSLs have been extensively studied because they are mainly produced by plants from the Brassicaceae family (Hopkins *et al.*, 2009). They are well-known toxic sulfured-containing metabolites, which hydrolysis by a myrosinase enzyme produces the volatile isothiocyanates (Björkman *et al.*, 2011). The GSL can be classified in three groups: aliphatic, aromatic and indole GSLs, according to their amino-acid precursor (Griffiths *et al.*, 2001). There are constitutive levels of GSLs in both roots and shoots, each dominated by aromatic and indole GSLs respectively, with however a higher concentration and diversity of GSLs in the roots, even though there are also GSL variation within roots (van Dam *et al.*, 2009). Feeding from an aboveground herbivore increased root GSL contents in both primary and secondary roots (Soler *et al.*, 2007). Jeschke *et al.* (2017) showed that GSLs had detrimental effects on herbivores, since they decreased their larval growth rate and increased their development time. However, generalist and specialist insects react differently to GSLs. Li *et al.* (2000) showed that both had a decreased larval growth when submitted to a diet with increasing concentrations of GSLs, but the impact was greater on the generalist, while the study of Arany *et al.* (2008) indicated that only the generalist had its weight negatively correlated to the GSL concentrations.

2.2.2. Indirect defenses

Indirect defenses correspond to either constitutive or inducible plant defenses that serve as cues to attract the natural enemies of the attacking herbivore (Aljbory and Chen, 2018). Such enemies can be predators or parasitoids².

Among constitutive indirect defenses, extrafloral nectar and food bodies are attractive food sources for parasitoids and predators (Mathur *et al.*, 2013), while domatias are plant sheltering cavities that they can use (Aljbory and Chen, 2018).

The most studied indirect and inducible defenses are volatile organic compounds (VOCs) and particularly herbivore-induced plant volatiles (HIPVs), which production increases

² Parasitoid: insect that lives in close association (inside or on) with its insect host and at the host's expense, and which sooner or later kills it.

following a herbivore attack, hence making the plant more attractive to parasitoids than a healthy plant (Van Poecke *et al.*, 2001). However, plants can also react to oviposition³ and consequently produce VOCs (Conti *et al.*, 2008). Several chemical groups are part of HIPVs: terpenes (Harmel *et al.*, 2007), green leaf volatiles (Scala *et al.*, 2013) and nitrogen-containing compounds (Aljbory and Chen, 2018). Part from the latter group, GSL-hydrolyzed isothiocyanates are produced by Brassicaceae when a plant is attacked (Textor and Gershenzon, 2009) and can either attract natural enemies or be detected by herbivores and act as repellent (Clavijo McCormick *et al.*, 2012). Apart from being parasitoid-attractive and herbivore-repellent, VOCs can also directly impact herbivore fecundity (Scala *et al.*, 2013) and act as priming agent to other plant tissues or neighboring plants, hence warning the surrounding of potential future attacks (Erb *et al.*, 2015). Lastly, VOC production can be variable depending on the plant suffering from single or dual herbivory and depending on the herbivory location (i.e. above or belowground, Pierre *et al.*, 2011).

2.2.3. Phytohormones: multitasking in biological interactions

Plant hormones or phytohormones are molecules produced by the plant in low concentrations. So far, recognized phytohormones are abscisic acid, auxin, brassinosteroids, cytokinins, ethylene, gibberellins, strigolactones, and jasmonic and salicylic acids (respectively JA and SA) and they promote either the plant growth or defenses, resulting into a trade-off between these two functions (Huot *et al.*, 2014). As they are multiple crosstalks between the different phytohormone pathways, most of them were found to play a role in regulating the plant defenses (Howe and Jander, 2008; Bari and Jones, 2009).

JA and SA are the most studied phytohormones, which pathways are activated upon insect herbivory and appear to be antagonist to each other, thus involved in a negative crosstalk (Okada *et al.*, 2015). Indeed, root and leaf GSLs were shown to respond to both JA and SA elicitations (Kiddle *et al.*, 1994; van Dam *et al.*, 2009; Zang *et al.*, 2015). Moreover, JA pathway is mainly activated by chewing insect while SA pathway is activated by phloem-sucking insect and gene expression associated to these pathways were found to increase following herbivory (Fürstenberg-Hägg *et al.*, 2013). Additionally, phytohormones also play a role for plant microbial communities. They can be involved in the regulation of mycorrhizal symbioses (Pozo *et al.*, 2015), and thus can shape the plant microbial communities, as JA- and SA- mutants of *Arabidopsis thaliana* had different leaf bacterial communities (Kniskern *et al.*, 2007).

³ Oviposition: action of laying eggs by a female insect using a specialized organ called ovipositor.

Altogether, it is not surprising to find that phytohormones can be implicated in interactions between plants, insects and microorganisms (see Zhang, 2017), by being modulated by plant-microorganism interactions against herbivorous insects or by insect-microorganism interactions against the plant (see section 2.5).

2.3. Plant-microorganism interactions

2.3.1. Plant microbial communities

Plants are organisms that live in interaction with their environment and other organisms such as microorganisms. Plenty of studies have been conducted on a given plant and a targeted microbial strain or species while in reality a plant interacts with more than one strain or species. Indeed, a plant is associated to communities of microorganisms, encompassing several groups of populations, themselves composed of several different species, interacting together and living in the same space at a given time. In this manuscript, microorganisms are studied and will be discussed at the community scale.

Roots are so far, one of the two most studied plant compartments for interactions with microorganisms. Various microorganisms live both in and on the roots (figure 3), corresponding to the endosphere and rhizoplane⁴ respectively (Vandenkoornhuyse *et al.*, 2015). Roots can be involved in intimate relationships with microorganisms. For example, the apparition of root nodules is a consequence of the mutualistic interaction between plant roots from the Fabaceae family and rhizobia from the *Rhizobium* genus, which are diazotrophic bacteria (Richardson *et al.*, 2009). These rhizobia are able to fix nitrogen from the atmosphere by converting it into nitrogenous compounds like ammonium that can be assimilated by the plant, in exchange for carbon-containing compounds resulting from photosynthesis. Root-fungi symbiosis, also called mycorrhiza present in most plants on Earth, can change the root morphology as well (Richardson *et al.*, 2009). In this case, the filamentous structures of the fungus (i.e. hyphae) invades the root compartment and either spread between the plant cells (i.e. ectomycorrhiza) or penetrate in these cells (i.e. endomycorrhiza). This feature will allow the plant to have access to water and nutrients from the soil, in exchange for carbon-containing compounds to the fungus.

⁴ Rhizoplane: plant habitat corresponding to the root surface.

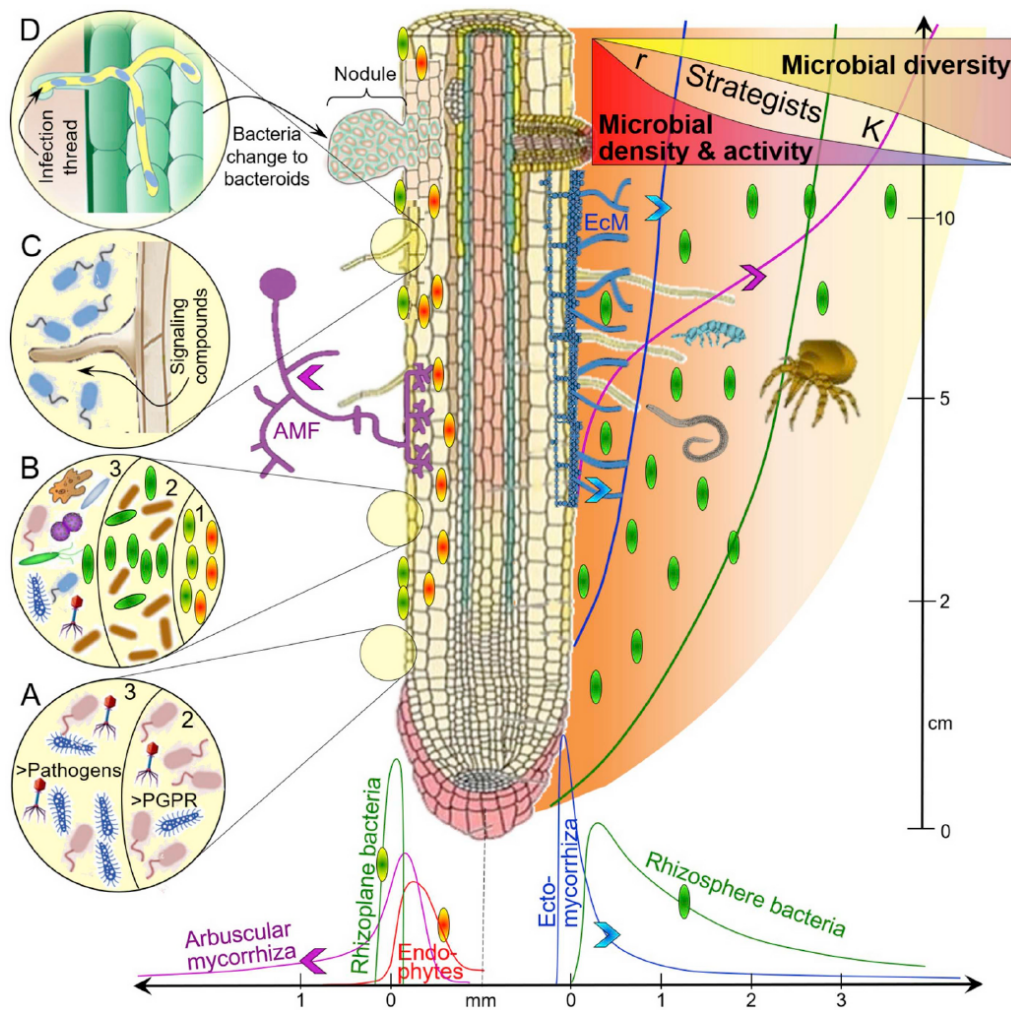


Figure 3 Distribution of life in the rhizosphere (from Kuzyakov and Razavi, 2019). The abundance of various microbial groups across (X axis at the bottom, in mm from the root surface) and along (Y axis at the right, in cm, not proportional) the young root is presented by continuous color curves. Microbial groups include: Arbuscular mycorrhiza (violet, AMF) and Ectomycorrhiza (blue, EcM); Endophytic, Rhizoplane and Rhizosphere bacteria (green). The gradients of microbial density, activity and diversity, as well as the dominance of r and K strategists are presented at the top right. The loupes magnify various processes and microbial distribution: A: higher density of plant growth promoting rhizobacteria (PGPR) compared to pathogens in 2) the rhizosphere and 3) reverse in bulk soil; B: abundance of various microbial groups 1) on the rhizoplane, 2) in the rhizosphere, 3) in the bulk soil; C: release of signaling compounds and attraction of rhizobia and other PGPR; D: infection of root hairs by rhizobia and formation of nodules. The numbers in the loupes on the left reflect: 1) rhizoplane, 2) the rhizosphere, 3) bulk soil. The schematic presentation of the abundance of individual microbial groups to the left or right of the root is made solely to avoid much overlapping of the curves. The overall life density is presented with orange shading on the right.

The second most studied compartment is the rhizosphere (Berendsen *et al.*, 2012). The notion of rhizosphere was first introduced by Lorenz Hiltner as the “soil influenced by roots” (Einflusssphäre der Wurzel) in 1904 (Hartmann *et al.*, 2008). Therefore, it corresponds to the thin layer of soil (i.e. a few millimeters wide) surrounding the roots. The rhizosphere was recognized for being a very dynamic zone since there are many exchanges between the plant and its environment (i.e. soil). Indeed, plants interact with many microorganisms that live in the rhizosphere such as protozoa, nematodes, arthropods, algae, archaea, viruses, fungi,

oomycetes or bacteria (figure 3-4A). The nature of their interaction is also variable, depending on whether a microorganism is pathogenic or mutualist (figure 4B).

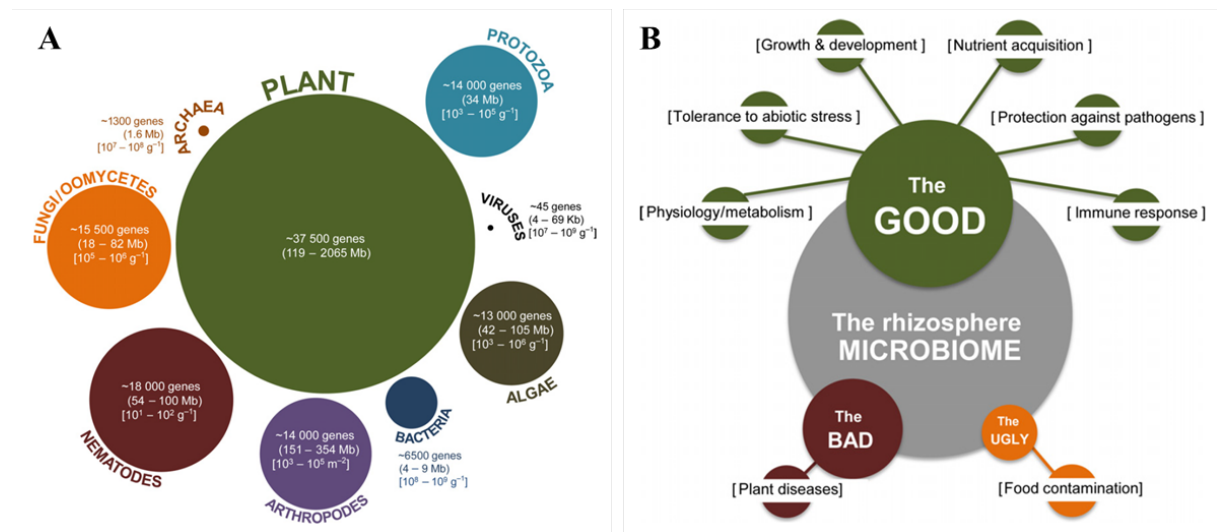


Figure 4 Overview of (micro)organisms present in the rhizosphere (A) and of their functions (B), from Mendes *et al.* (2013)

Microorganisms have colonized all plant compartments. The microbiota of the seeds has been gaining attention (Barret *et al.*, 2015), as well as the one from the phyllosphere, corresponding to microbial habitat of the leaves (Vacher *et al.*, 2016).

2.3.2. Origins, transmission and modulation of microorganisms

One of the primary source of inoculum for the plant comes from the vertical transmission, where seed microbiota can be passed down to seedlings (Shade *et al.*, 2017). The study of Rochefort *et al.* (2019) demonstrated that genotype and environment can contribute to shaping the seed microbial communities. Similarly, host plant genotype as well as age were found to affect root and leaf microbial communities (Wagner *et al.*, 2016), while rhizosphere microbiota is actively modulated by plant development (Chaparro *et al.*, 2014).

Another source of inoculum for the plant is the soil, qualified by Vandenkoornhuyse *et al.* (2015) as a “seed bank for root microbiota”. Many factors can affect the soil and thus strongly drive soil microbial communities, which will consequently modify the microbial communities associated to the plant. The soil type differently shapes root microbiota as soil from several collection sites will be characterized by a different pedology and climate (Bulgarelli *et al.*, 2012; Lundberg *et al.*, 2012). Agricultural practices also have an important influence on the plant microbiota through soil manipulation. Several studies have focused on the effect of tillage and found contrasting results. Janušauskaite *et al.* (2013) showed that the

absence of tillage reduced soil microbial diversity, while Dong *et al.* (2017) found that no tillage was associated with an increase of bacterial diversity. As soil physicochemical properties are greatly changed by this practice, it is not surprising that such effect echoes on the plant microbiota (Rathore *et al.*, 2017). Moreover, Hartmann *et al.* (2015) demonstrated that organic farming also tended to increase soil bacterial and fungal richness. Several studies have assessed the effects of amendments on soil microbiota. While the crop production was lower with organic amendments, the soil became richer and more diverse in term of microorganisms (Francioli *et al.*, 2016; Cesarano *et al.*, 2017).

Plants can also acquire microorganisms from other organisms, like herbivorous insects vectoring plant pathogens from one plant to another (Whitfield *et al.*, 2015; Flury *et al.*, 2018). The most studied case is aphids vectoring plant viruses through their mouthpart (i.e. stylet), as also do whiteflies, leafhoppers and thrips.

2.3.3. Plant microbial management and services

To form symbiosis with the plant, microorganisms can be attracted and recruited by the roots (figure 5). Such attraction is due to the plant chemistry, as the roots secrete organic compounds into the soil, corresponding to the rhizodeposition⁵. It was estimated that roots secrete 5 to 21% of the photosynthetic carbon outcomes under the form of sugars, amino acids and secondary metabolites (Huang *et al.*, 2014). These secretions are called root exudates⁶, and are mediators in the rhizosphere between the plant and microorganisms. Indeed, application of in vitro-generated root exudates differently shaped soil fungal communities (Broeckling *et al.*, 2008) and bacterial communities (Badri *et al.*, 2013). In the case of both root nodules and mycorrhiza, resulting from root-microorganism interactions, the plants attract their symbionts with carbohydrates (Kiers *et al.*, 2011). The authors showed that the more carbon is given to fungi by the plant, the more phosphorus is given to the plant by the fungi, and that plants had the capacity to reward their symbionts when receiving phosphorus. Similarly, phytohormones also have control over the mycorrhiza development, by either inhibiting or enhancing the fungal spread in the roots or arbuscule formation (Pozo *et al.*, 2015). Lastly, Schulz-Bohm *et al.* (2018) highlighted the role of plant VOCs in microbial recruitment where bacteria were attracted to

⁵ Rhizodeposition = All of the organic carbon derived from roots that is lost into the soil. This includes compounds lost from live roots (e.g. root exudates, root cap cells, border cells), root symbionts (e.g. mycorrhizas, N₂ fixing bacteria, dark septate fungi) and also compounds lost from senescing and dead roots (i.e. root turnover). Definition from Oburger and Jones (2018)

⁶ Root exudates = Soluble organic compounds lost from the surface of living root cells into soil including low molecular weight (MW; <1000 Da; e.g. sugars, organic acids, phenolics, vitamins) and high MW compounds (> 1000 Da; e.g. enzymes, mucilage). Definition from Oburger and Jones (2018)

VOCs emitted by the plant, but only specific bacteria were recruited upon a fungal pathogen infection.

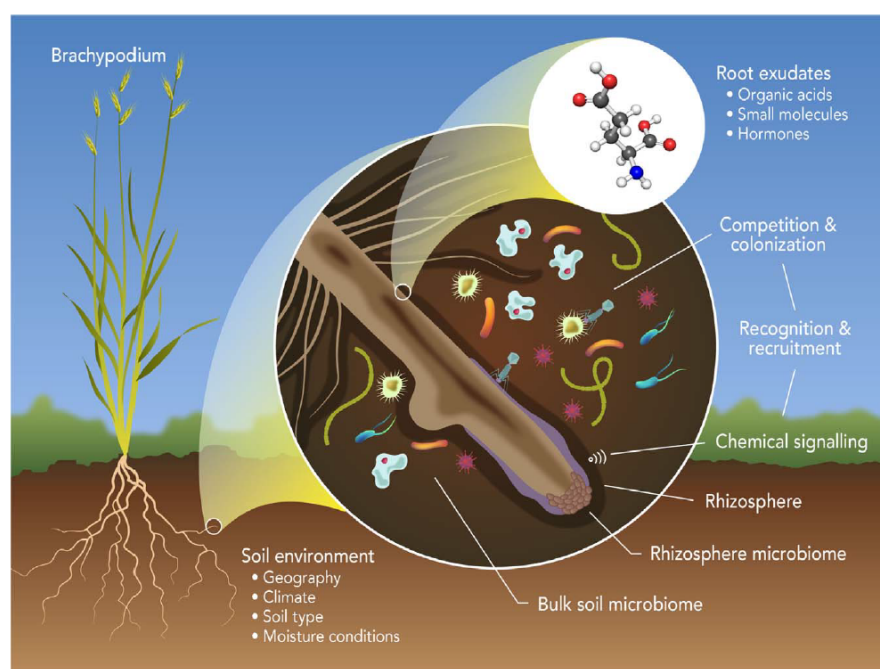


Figure 5 Overview of the rhizosphere dynamic (White *et al.*, 2017)

As various microorganisms can be recruited by the plant through root exudates, phytohormones or VOCs, they provide advantages to their host. Plant-microorganisms interactions improve the plant growth and development like the well-known plant growth promoting rhizobacteria, also called PGPR (Richardson *et al.*, 2009). They can enhance the root growth, which will increase the plant nutrition, as the symbioses with either rhizobia or mycorrhizal fungi, which improve respectively the plant nitrogen and phosphorus nutrition in exchange for carbohydrates. Other microorganisms can also increase the plant nutrition like bacteria of non-leguminous plants and other fungi for nitrogen, or such as *Pseudomonas* with sulfur mineralization as an underlying mechanism of plant growth promotion (Jacoby *et al.*, 2017). Microorganisms also work in synergy like rhizobia and mycorrhiza, which combination resulted in higher fixed nitrogen content and higher seedling establishment compared to being tested separately or to control (van der Heijden *et al.*, 2016). Moreover, microbial symbiosis was shown to increase both plant biodiversity and productivity (Van der Heijden *et al.*, 1998; van der Heijden *et al.*, 2008, 2016). Finally, a very interesting advantage conferred by microorganisms to their host plants is the capacity to protect their host against bioaggressors like a pathogen (Mendes *et al.*, 2011). Indeed, some soils associated with bacteria from the γ -Proteobacteria class have shown the particularity to have a disease-suppressive activity and

plants growing on this soil displayed less symptoms to a fungal root pathogen. Several recent studies focused on the role of soil and rhizosphere microbiota on the plant resistance against herbivorous insects (see section 2.5).

2.4. Insect-microorganism interactions

As plants, insects can harbor many types of microorganisms, in less abundance though. They can be either harmful such as bacterial, fungal pathogens and viruses or beneficial (and then commonly referred to as symbionts), or even both.

2.4.1. Characteristics of insect-microorganism symbiosis

Beneficial insect symbionts are classified in two categories. An “obligate or primary” symbiont is a microorganism, which is indispensable to its host to complete a successful development and reproduction. A famous and largely studied example would be the bacteria *Buchnera aphidicola*, essential to their aphid host survival (Douglas, 1998). These bacteria provide essential amino acids to their host and a lack of *B. aphidicola* leads to the development of smaller aphid adults, which will produce fewer or no offspring (Douglas, 1998). If not obligate, a symbiont can be “facultative or secondary”, therefore not required by the host as it can achieve successful development and reproduction without it. Even if they are not obligate, facultative symbionts can contribute to their host fitness by conferring different kinds of advantages such as increased resistance to heat shock or improvement of insect fecundity (Oliver *et al.*, 2010; Guo *et al.*, 2017); protection against natural enemies like parasitoids, nematodes or pathogens (Oliver *et al.*, 2014). Recent findings have also highlighted the capacity of insects to deal with the plant defenses through their symbionts (see section 2.5).

Both obligate and facultative symbionts can be distinguished according to their location, the area or compartment they colonize. Ectosymbiontes are symbionts that live on the external or internal surface (i.e. cuticle) of their host. For example, the cuticle of ant workers are colonized by Actinobacteria, which form a whitish coating (de Souza *et al.*, 2013). Endosymbiontes are symbionts that live inside their host or within its cells, and in the latter case the symbiosis is said to be intracellular, contrasting with extracellular. For instance, symbiosis between aphids and *B. aphidicola* has evolved to the point of developing specialized aphid cells (i.e. bacteriocytes) where the symbiont is located (Douglas, 1998). *Wolbachia* is another well-known endosymbiont that lives inside its host cells (Werren *et al.*, 2008). Sacchi

et al. (2010) found out that *Wolbachia* could reside in bacteriocysts present in *Drosophila* ovaries. Otherwise, symbionts were recognized to be able to reside and circulate in insect hemolymph, which corresponds to the fluid that flows through the whole body (Blow and Douglas, 2019).

2.4.2. Insect microbial transmissions

Insects acquire their symbionts through two ways of transmission.

The first way is vertical transmission that corresponds to symbionts transmitted from the mother to its offspring. For insects, it is largely admitted that the mother can transmit symbionts through colonization of the eggs or embryos (Moran and Dunbar, 2006). However, the latter study showed that paternal transmission in aphids was possible and detected the presence of the transferred symbionts in the male testes and accessory glands (figure 6A). In these cases, transmission occurs internally. External transmission can also take place as demonstrated by the interesting method of the stinkbug (*Megacopta punctatissima*). This insect species harbors its obligate symbiont in the cavity of crypt-bearing posterior midgut and when adult females lay eggs on their host plant, small brownish particles are always deposited under the egg mass (Hosokawa *et al.*, 2007). The particles encase a copious amount of the symbiont inside, and hatchlings from the eggs orally acquire the symbiont from the capsule (figure 6B).

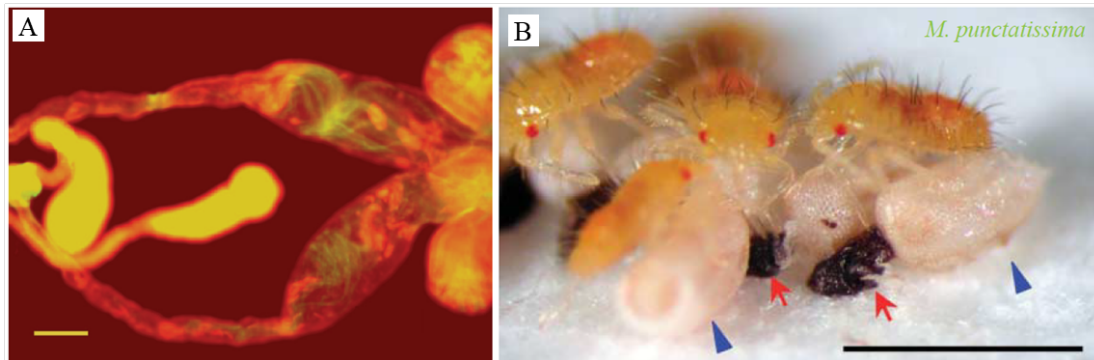


Figure 6 Localization of vertically-transmitted insect symbionts. (A) Localization of symbionts within the aphid male reproductive system by using FISH with probe matching the 16S rRNA sequence of *Regiella insecticola* (green and bright yellow) and with propidium iodide DNA counterstain (red). The testes are on the right; the greatest concentration of symbionts is within the accessory glands, at the center and left. Scale bars, 0.1 mm. (Moran and Dunbar, 2006). (B) Newborn nymphs of *M. punctatissima* probing capsules for symbiont acquisition. Arrows and arrowheads indicate symbiont capsules and eggshells, respectively. Scale bars, 1 mm. (Hosokawa *et al.*, 2007).

The second way is horizontal transmission that corresponds to symbionts acquired from other organisms or the environment. It should be noted that a symbiont acquired horizontally is not necessarily vertically transmitted to the host offspring, as the symbiont must priorly adapt

to its new environment by dealing with the host immune system. Horizontal symbiont acquisition can occur through coprophagy, for example newly born offspring can obtain symbionts by probing on conspecific feces (Salem *et al.*, 2015). Le Clec'h *et al.* (2013) have demonstrated that *Wolbachia* could be transmitted through predation and cannibalism. Therefore, diet and water can be used to transfer or modify gut microbiota (Mason *et al.*, 2014). Moreover, phylogenetic analyses have allowed scientists to assess horizontal transmissions that took place in the past between related (i.e. through trophic interactions) or unrelated species. Binetruy *et al.* (2019) have revealed that the bacterium *Spiroplasma ixodetis*, which is a maternally inherited endosymbiont of ticks, was horizontally transmitted several times in the past between ticks and other arthropod species like flies and aphids. Also, transmissions between hosts and parasitoids under field conditions were studied by Qi *et al.* (2019), which showed that *Rickettsia* and *Wolbachia* were indeed transferred between whiteflies and their parasitoids. Finally, insects can acquire symbionts from their environment such as the soil (Kikuchi *et al.*, 2012) and/or the plant, like the caterpillar *M. brassicae*, which microbiota was found to be highly similar to that of its host plant (Hannula *et al.*, 2019).

2.5. Tripartite interaction between plant, insect and their associated microorganisms

Both plants and insects harbor microorganisms, which confer advantages to their hosts. During the past two decades, several studies have highlighted the fact that these microorganisms can impact the other interacting party: plant microbiota on insects and insect microbiota on plants.

2.5.1. Plant microbial teamwork against insect herbivory

Entomopathogens, or organisms with a detrimental effect on insects, have been identified to be potential agents of biological control. Klingen *et al.* (2002) have attempted to list known and suspected entomopathogenic species that were found in Norway, among which there were mostly fungi (*Cordyceps*, *Beauveria*, *Fusarium*, *Metarhizium*, *Entomophthora*), but one bacterium species (*Bacillus thuringiensis*) and several viruses and nematodes. Identified fungi were associated with hymenopteran, coleopteran and hemipteran, but mainly with lepidopteran, dipteran insects. In Czech Republic, Kubátová and Dvůrák (2005) also led a sampling campaign to demonstrate that insect cadavers (lepidopteran, dipteran and

hymenopteran) collected from underground shelters were colonized by fungi, including some that are known to be entomopathogenous (*Beauveria*, *Conidiobolus*, *Cordyceps*, *Engyodontium*, *Hirsutella*, *Lecanicillium*, *Paecilomyces* and *Simplicillium*).

Cordyceps and *Beauveria* genera seem to be recurrent fungal entomopathogens. Indeed, it was observed that *Cordyceps* could generate stroma (i.e. agglomeration of hyphae), showing that it can grow on nymphs and adults of the click beetle, *Campsosternus auratus* (Zhang *et al.*, 2004). Bacteria like *B. thuringiensis* can also act as a natural biological pesticide as it was detected in the soil, plant tissues and also dead insects. This bacteria colonization pathway was identified using green fluorescent proteins, where plant roots took up the bacteria from the soil, which migrated up to the leaves and entered insects when they fed on the plant (Monnerat *et al.*, 2009). In this study, the lepidopterans *Spodoptera frugiperda* and *Plutella xylostella* larvae were sensible to *B. thuringiensis* and died after feeding on *B. thuringiensis*-treated leaves. Such toxicity comes from the crystal proteins produced by the bacterium and becomes harmful once in the insect midgut (Höfte and Whiteley, 1989). Similarly, *Beauveria* and *Metarhizium* are entomopathogenic fungi, that were also identified as being plant endophytic colonizers (Barelli *et al.*, 2016).

Microorganisms have been found to affect insects through their host plant by modulating the plant defensive mechanisms. For instance, microorganisms use the plant phytohormones to impact herbivorous insects. Indeed, the root-colonizing rhizobacteria *Pseudomonas fluorescens* negatively affect the weight of the generalist caterpillar *Mamestra brassicae* (i.e. the cabbage moth) but that of the specialist *Pieris brassicae* (i.e. the large white butterfly) through the JA pathways (Pangesti *et al.*, 2015a). Microorganisms can also modulate plant VOC emission in order to make the plant more attractive to parasitoids. Pangesti *et al.* (2015b) showed that *P. fluorescens* could increase the emission of certain VOCs while decreasing others like terpenes, thus attracting parasitoid wasps to plants infested by the generalist *M. brassicae*. It seems that microbial regulation of VOCs is linked to phytohormonal pathways. For example, the reduction of wounds inflicted by the fall armyworm (*Spodoptera frugiperda*) on maize seemed to be caused by the VOCs produced by the fungus *Trichoderma atroviride* (Contreras-Cornejo *et al.*, 2018). The authors also observed that this fungus increased both JA proportion and terpene emission, and this VOC is known to be regulated by the JA pathway.

So far, many studies have focused on the effects of a single strain or species, which were able to modulate the plant defenses and impact herbivorous insects as previously shown. Recently, more integrative studies have emerged and highlighted the capacity of several microbial species and even whole community to affect insects by modifying plant defenses. For example, Pangesti *et al.* (2017) demonstrated that despite two strains of *P. fluorescens* being antagonist to each other, their antagonism did not affect the plant growth promotion and the same negative impact on *M. brassicae* was observed upon both single or co-inoculation of the strains. On a more complex level, the inoculation of whole soil communities (nematodes or microorganisms) was found to influence the number of aphids developing on the plant, but could not be linked to the measured plant chemistry, like amino acids and GSLs (Kabouw *et al.*, 2011). However, Hol *et al.* (2010) showed that changes in plant chemistry could be linked to soil microbial communities. The authors manipulated the soil with dilutions in order to obtain three soils with a decreasing microbial diversity gradient. Plants growing on more diverse soil microbial communities strongly affected aphids, which were smaller on these plants which produced higher contents of secondary metabolites (i.e. GSLs) and had lower contents of primary metabolites (i.e. amino acids). In this case, the reduction of aphid size can be assumed to be caused by the increase of plant defenses, itself induced by the presence of diverse soil microbial communities. Moreover, removal of microorganisms from the soil definitely has a drastic negative effect on the plant and promotes insect development. The study of Hubbard *et al.* (2019) demonstrated that the filtration of a soil inoculum permitted the removal of microorganisms and plants growing on this inoculum had more aphids and leaf damages than plants grown on a non-filtered soil inoculum.

Several microorganisms have been commercialized in order to be used as agents of biological control against insects. For instance, *B. thuringiensis* is the most known and probably the most used in commercialized insecticides, referred as to “Bt” (Lacey *et al.*, 2015). Similarly, the *B. bassiana* species was commercialized under the Naturalis® product and was tested on tomato leaf miner (*Tuta absoluta*) that had lower longevity when fed on a leaf, treated the day before with Naturalis® (Klieber and Reineke, 2016). Insects that died during the bioassays displayed external morphological features of fungal colonization. However, the commercialization of whole microbial communities has yet to be achieved.

2.5.2. Interference of insect microbial symbionts in plant defenses

Several recent studies have demonstrated the capacity of insect symbionts to manipulate, disturb or suppress the plant response to insect herbivory (Zhu *et al.*, 2014).

Insect symbionts can manipulate plant phytohormones in order to promote their host development. For instance, the leaf mining lepidopteran caterpillar (*Phyllonorycter blancardella*) was able to increase its nutritional intake thanks to its endosymbiotic bacteria which modifies the cytokinin phytohormone to maintain green and palatable spots (also referred as to green islands) on senescent leaves (Kaiser *et al.*, 2010; Body *et al.*, 2013).

Insect symbionts can reduce the plant direct defenses. Indeed, the study of Chung *et al.* (2013) showed the presence of the *Stenotrophomonas*, *Pseudomonas*, and *Enterobacter* genera in the Colorado potato beetle (*Leptinotarsa decemlineata*) larvae decreased plant defenses (i.e. polyphenol oxidase activity), and modified gene expressions associated to the JA and SA pathways. Similarly, Ceja-Navarro *et al.* (2015) demonstrated that gut symbionts were necessary to the coffee berry borers (*Hypothenemus hampei*) in order to assimilate caffeine, which is an alkaloid (i.e. toxic secondary metabolites). Among the assessed bacteria of *H. hampei*, *Pseudomonas fluva* was the only bacterium that harbors a gene coding for a caffeine demethylase and the presence of this bacterium on antibiotic-treated insect was enough to degrade caffeine. Therefore, these symbionts degrade caffeine so it could be assimilable and eatable for their host. Lastly, a study led by Mason *et al.* (2014) showed that larvae of the gypsy moth (*Lymantria dispar*) could keep developing by using microorganisms of foliar origin to counter plant secondary metabolites (i.e. phenolic glycoside) and they identified the genus *Acinetobacter* as having the capacity of reducing these plant defenses.

Insect symbionts can also disturb plant indirect defenses. In natural conditions, larvae of the mountain pine beetle (*Dendroctonus ponderosae*) are submitted to high levels of terpenes (i.e. VOCs) produced by their host plant, but they still overcome it. By isolating bacteria and fungi from these insects, Boone *et al.* (2013) showed that the bacteria *Serratia marcescens* had the strongest impact on monoterpenes and could reduce their concentrations. Similar results were obtained for diterpenes, which concentration dropped with the presence of *S. marcescens* and *Bacillus vesicularis*.

Interestingly, insects can also acquire insecticide resistance through microorganisms. Soil is a very well-known microbial reservoir for plants (Vandenkoornhuyse *et al.*, 2015), but less recognized for insects while it can also be important. Kikuchi *et al.* (2012) showed that the bean bug *Riptortus pedestris* acquires its symbiotic bacteria of the genus *Burkholderia* from the

soil during its development. However, some *Burkholderia* strains present in the soil have developed the capacity to degrade insecticide molecules and can be acquired by *R. pedestris*. Kikuchi *et al.* (2012) demonstrated that this symbiosis improved the fitness and survival of the bean bug when reared on soybean seeds dipped in insecticide, hence showing the acquired insecticide resistance from *Burkholderia*.

2.6. Agricultural context

Insect herbivory can cause massive damages to plants, causing their death in case of high insect infestation. Injuries from insect herbivory can also cause the plant to be more susceptible to diseases as wounded tissues can become a pathway for pathogens to invade, infect and colonize the attacked plant. On a larger scale, insect herbivory can provoke great damages to plant production such as agroforestry (Rao *et al.*, 2000) and agriculture (Crawley, 1989). In the latter case, crop fields can suffer to the point of generating important yield losses for farmers. They are for example estimated to be up to several hundreds of millions dollars annually in North America (Ahuja *et al.*, 2010).

To avoid such economical loss, insecticides were the most common used method. Besides being an important financial investment for farmers, these products have shown to be greatly harmful to the environment and non-targeted organisms on one hand (Paoletti and Pimentel, 2000; Devine and Furlong, 2007) and to human health on the other hand (Bjørling-Poulsen *et al.*, 2008). Therefore, the field of agriculture has been submitted to increasing political regulations during the past decade, such as the French “Ecophyto” plan (<https://agriculture.gouv.fr/ecophyto>), which urges to reduce the use of insecticides. Consequently and knowing that the creation of insect-resistant varieties are yet to be achieved for many crops, the challenge is to develop alternative methods to control herbivorous insects in a context of a more eco-friendly and sustainable farming.

The manipulation of biological interactions to control herbivores seems to be promising, especially through the use of organism-produced molecules or the organisms themselves. Indeed, plant-insect interactions have been largely studied to better understand the plant defensive mechanisms and in order to enhance the plant defensive capacity against herbivorous insects. The beneficial role of microorganisms have been recognized to improve both plant and insect health, but the integration of microorganisms in plant-insect interactions is still recent. Such development is linked to the evolution of technologies, tools and methodologies for both

chemical and molecular assessment, and to the “holobiont” concept that implies a breakthrough of more integrative studies as we entered the “omic era” (figure 7).

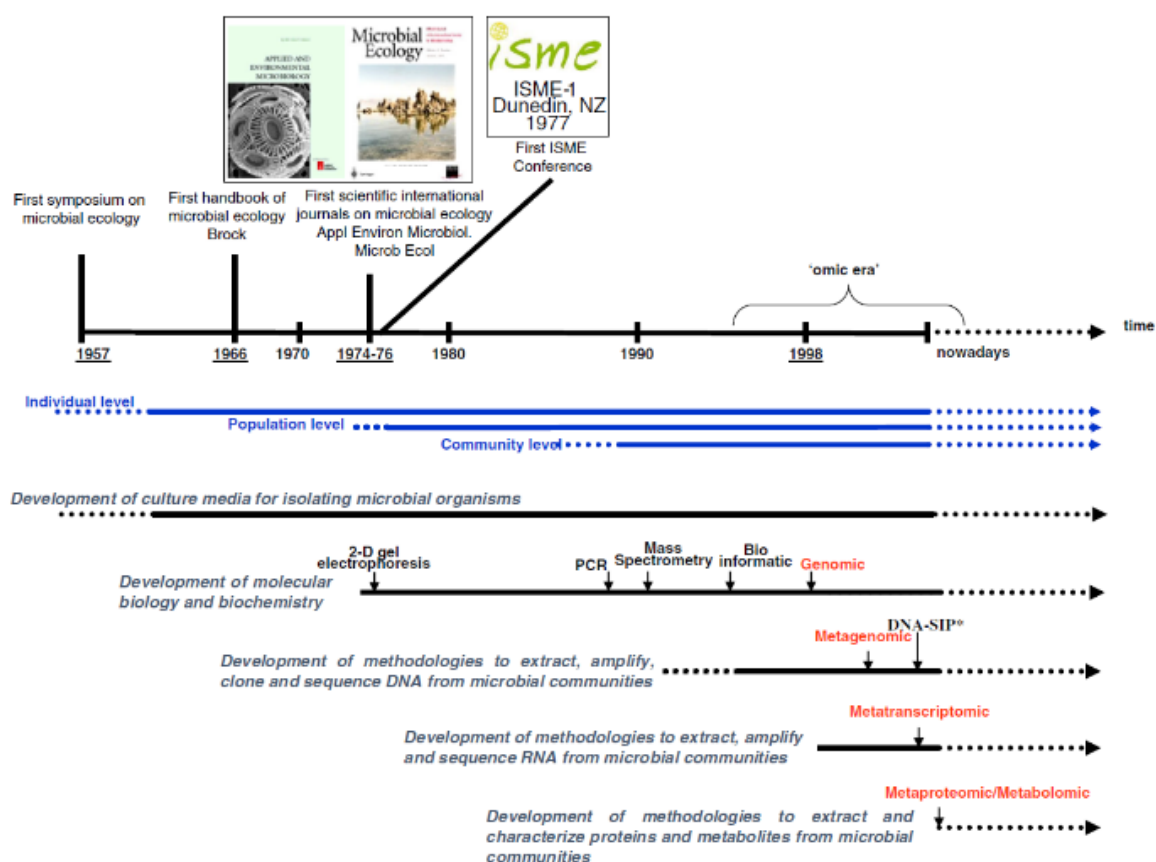


Figure 7 Evolution of technologies for metabolite and microorganism assessments (Lemanceau *et al.*, 2015).

2.7. Technological and analytical advances for microbial communities

For years, microorganisms were studied using culture-dependent approaches by growing them in Petri dish. However, only a small portion of microorganisms are cultivable. During the past decade, great progresses were realized to develop culture-independent approaches and now, the New Generation Sequencing (NGS, e.g. Illumina MiSeq) and molecular tools (e.g. 16S rDNA, 18S rDNA and *gyrB* primers) have allowed us to unravel the diversity of both cultivable and non-cultivable microorganisms at the finest level of taxonomy.

To assess the diversity of plant or insect microbial communities, alpha- and beta-diversity measurements are often used, even though these notions were first employed in ecology of plant communities (Whittaker, 1972). Alpha-diversity is associated to several indices. One of them is species richness, corresponding to species diversity or more precisely the number of observed species, or OTUs (Operational Taxonomic Units) and ASVs (Amplicon Sequence Variants) in the communities as later mentioned in the manuscript. Another diversity

index is the Shannon index, defined as “the relative evenness or equitability of the importance values through the whole sequence” (Whittaker, 1972). By measuring how species are distributed in a sample, the Shannon index gives information about: species distribution among each other, “how evenly the individuals in the community are distributed over the different species” (i.e. evenness, Heip *et al.*, 1998), and whether there are dominant species in the community. Beta-diversity can be defined as a “change in species composition of communities [...] or biotic change along environmental gradients” and therefore can also be referred as to the structure of microbial communities (Whittaker, 1972).

3. Main objectives of the thesis

Many studies about soil or root microorganisms being potential agents of insect biological control, have already focused on one microbial strain or species. While the effects of such microorganisms have been studied on aboveground herbivores, which development could be altered by plant defenses, knowledge about the effects of whole microbial communities on belowground herbivores is still scarce. Therefore, it raises the questions of i) whether soil microbial communities could negatively affect a belowground herbivore through potential modification of plant defenses and ii) how the herbivore influences back the plant chemistry and microbial communities.

Moreover, symbionts strongly influence their insect host phenotype, and in some case provide protection against plant defenses. Thus, it is essential iii) to evaluate insect bacterial communities in different contexts to understand how bacteria react to insect host manipulation or how bacteria are transmitted to their insect host.

In this manuscript, we will focus on these three objectives, associated to the interaction between oilseed rape (*Brassica napus*), which is a Brassicaceae crop plant, and the cabbage root fly (*Delia radicum*), a specialist root herbivore of Brassicaceae. We will assess the influence of soil microbial communities and of root herbivory in the interactions between plant, insect and their associated microbiota and we will evaluate insect bacterial communities, using the latest NGS technology (i.e. Illumina MiSeq) and molecular tools (i.e. 16S, 18S, *gyrB* primers).

4. Biological models

4.1. The plant holobiont: oilseed rape (OSR, *Brassica napus*)

4.1.1. Taxonomy and identification

Oilseed rape (“OSR”) *Brassica napus* subsp. *napus* is a crop, hence a cultivated plant from the Brassicaceae family (Plantae, Brassicales), formerly known as Cruciferae family. Brassicaceae consists of 49 tribes, 321 genera and 3660 species (Koch and Marhold, 2012), including *Arabidopsis thaliana*, a commonly used and well-known research model, while the genus *Brassica* comprises 159 species (Ahuja *et al.*, 2010). In the literature, OSR is associated to many different names such as rapeseed, rape or canola, with the latter term mainly used in Canada. This crop is an amphidiploid that resulted from the interspecific spontaneous hybridization between a cabbage *Brassica oleracea* L. and a turnip rape *Brassica rapa* (Downey and Rimmer, 1993). There are different types of OSR depending the season it is sown: winter OSR, semi-winter OSR and spring OSR. In this manuscript, only winter OSR was used.

4.1.2. Distribution and economic impact

Oilseed rape is a crop plant which has a massive agronomic importance (Ahuja *et al.*, 2010) as it is cultivated worldwide (figure 8A) and Europe was the first OSR producers in 2017 with 26 millions of tons (figure 8B). Meanwhile, France was fourth in the top 10 countries producing this crop with 5 millions of tons (figure 8C). Since the 60’s, the surface of cultivated oilseed rape has been rising, as well as its production and yield (figure 8D-E). Oilseed rape seeds can be used to make pellets or vegetative oil, for livestock and human alimentation respectively, as well as brut oil used as biodiesel, which values reach millions of euros every year in France (figure 8F). Hence, OSR has also a major economical importance and this implies that farmers spend time and money in order to protect their crops against bioagressors (i.e. pathogens, insects). Since the 90’s, the amount of total insecticides (i.e. chlorinated hydrocarbons, organo-phosphates, carbamates insecticides, pyrethroids) used in France has decreased but seems to increase again since 2010 (figure 8G).

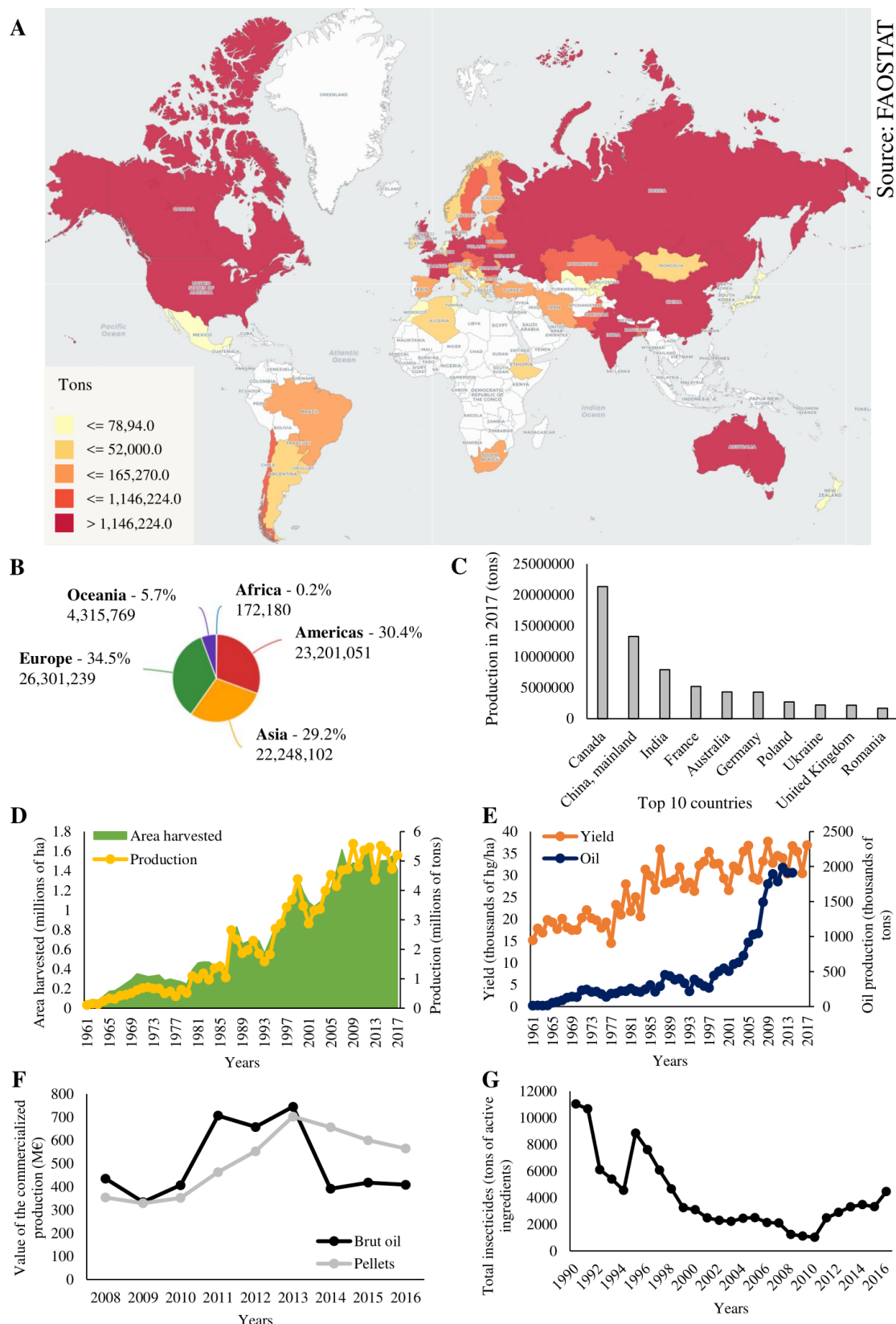


Figure 8 Oilseed rape crop over the world and in France. Production quantities of oilseed rape (in tons) in 2017 all over the world (A), in all 5 continents (B) and in the top 10 countries (C). Evolution of the area harvested, the crop production (D), the yield and the oil production (E), the financial outcomes (F) associated to oilseed rape crop in France and of total pesticides used in France (G). Data was obtained from the database of the Food and Agriculture Organization of the United Nations (<http://www.fao.org/faostat/en/>) and from the Agreste database (<http://agreste.agriculture.gouv.fr>).

4.1.3. Development and insect pests

The biological cycle of winter OSR starts in September with sowing and spends winter under a vegetative state. During spring, plants elongate and form bud that turn into yellow flowers, which transform into pods containing the new formed seeds (figure 9). Each stage of this cycle and plant compartments are susceptible of being attacked by different pathogens or herbivorous insects (Bonnemaïson, 1965; Ahuja *et al.*, 2010). Insects can seriously damage the plants when they reproduce or feed on the plants, and such insects are call herbivorous or phytophagous insects, or shortly herbivores. So far, the most commonly used method against these insects was chemical products like pesticides or insecticides, but now farmers have to reduce the use of pesticides and move on to a more eco-friendly and sustainable agriculture.

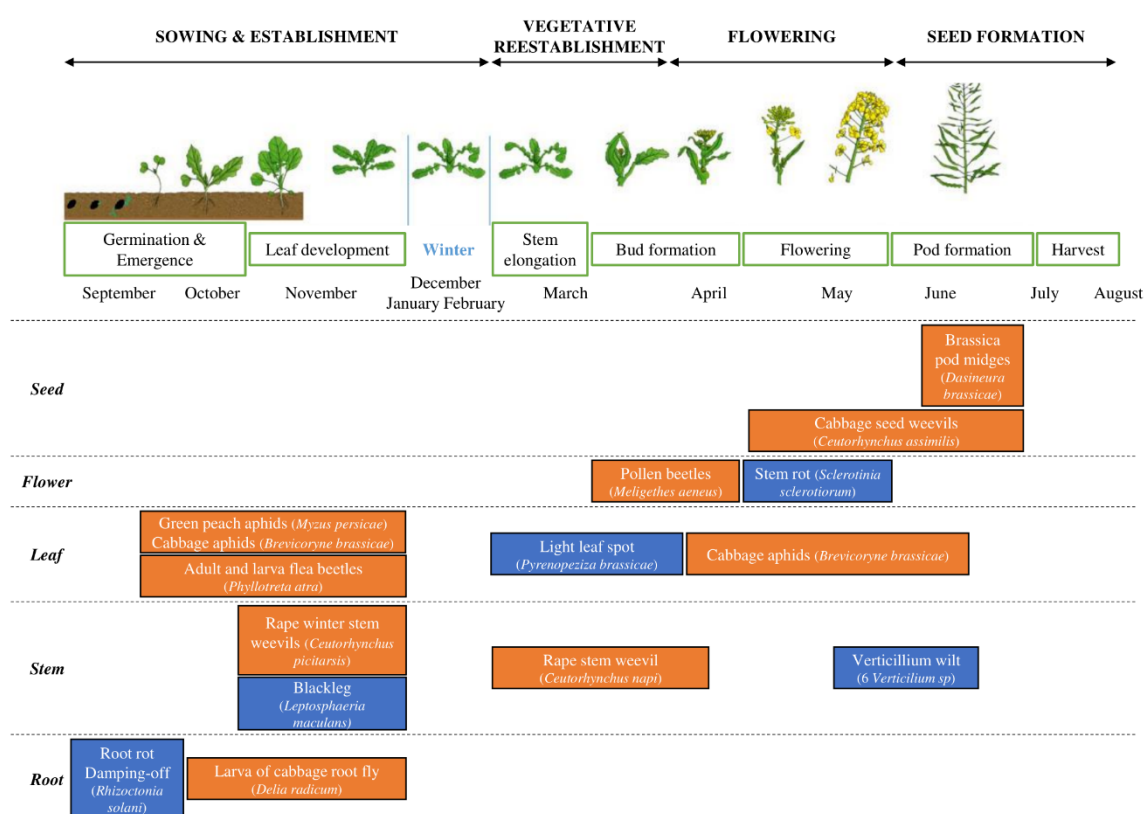


Figure 9 Biological cycle of winter oilseed rape (*Brassica napus*) and its main bioaggressors. Occurrence of main bioaggressors is represented by the colored boxes, in orange and blue respectively for insects and diseases. Adapted from Terres Inovia and <http://www.semences-de-colza.fr>

4.1.4. Microbial ecology of *B. napus*

It should be kept in mind that plants from the Brassicaceae family do not develop symbiosis with mycorrhizal fungi or rhizobia, which can bring advantages to their hosts in terms of nutrition or protection against insect herbivory as discussed above.

When analyzing Brassicaceae microbiota, many studies used the well-known biological model *Arabidopsis thaliana* and few have focused on crop plants. We chose OSR as this plant is a Brassicaceae crop cultivated worldwide. Several studies have identified beneficial microorganisms associated with *Brassica* species, which were listed by Card *et al.* (2015). The microorganisms associated to *B. napus* can enhance plant growth promotion, be antagonist or inhibit pathogens or provide resistance to heavy metals (table 1). But only one fungus (*Metarhizium anisopliae*) was reported to inhibit an aboveground herbivorous insect attacking *B. napus*.

Table 1 Endophytic bacterial and fungi associated with *B. napus* and their beneficial activity (modified from Card *et al.*, 2015). Bold writing corresponds to plant microorganisms beneficial against insects.

Type of bacteria	Microbial species	Beneficial activity
Gram-positive bacteria	<i>Bacillus</i> spp.	Heavy metal resistance, antagonism towards <i>Leptosphaeria maculans</i> & <i>Sclerotinia sclerotiorum</i>
	<i>B. amyloliquefaciens</i>	Antagonistic potential against <i>Xanthomonas campestris</i> & a number of fungal pathogens
	<i>B. endophyticus</i>	Antagonistic towards a number of fungal pathogens
	<i>B. subtilis</i>	Growth promotion, antagonism towards <i>X. campestris</i> , <i>S. sclerotiorum</i> & <i>Colletotrichum higginsianum</i>
	<i>Microbacterium</i> sp.	Heavy metal & antibiotic resistance, plant growth promotion under heavy metal-contaminated soils
	<i>Paenibacillus polymyxa</i>	Antagonism towards <i>Verticillium longisporum</i>
	<i>Alcaligenes</i> sp.	Inhibition of <i>Verticillium dahliae</i>
Gram-negative bacteria	<i>Azorhizobium caulinodans</i>	Potential for plant growth promotion
	<i>Enterobacter</i> sp.	Inhibition of <i>V. dahliae</i> & plant growth promotion
	<i>Pseudomonas chlororaphis</i>	Inhibition of <i>V. dahliae</i>
	<i>P. fluorescens</i>	Heavy metal & antibiotic resistance, plant growth promotion & antagonism towards <i>X. campestris</i>
	<i>Rhanella</i> sp.	Plant growth promotion and heavy metal uptake
	<i>Serratia proteamaculans</i>	Inhibition of <i>V. dahliae</i>
	<i>S. plymuthica</i> *	Improved seed germination
	<i>Stenotrophomonas</i> spp.	Inhibition of <i>V. dahlia</i>
Fungi	<i>Alternaria alternata</i>	Plant growth promotion
	<i>Aspergillus flavipe</i>	Reduced disease caused by <i>Sclerotinia sclerotiorum</i>
	<i>Chaetomium globosum</i>	Anti-fungal activity towards a number of bacterial & fungal pathogens
	<i>Fusarium oxysporum</i>	Antifungal activity towards <i>S. sclerotiorum</i> & <i>Botrytis cinerea</i>
	<i>F. tricinctum</i>	Plant growth promotion
	<i>Leptosphaeria biglobosa</i>	Plant growth promotion and antifungal activity towards <i>S. sclerotiorum</i>
	<i>Metarhizium anisopliae</i>	Inhibited the larvae of <i>Plutella xylostella</i>

*Rhizosphere

To my knowledge, Gkarmiri *et al.* (2017) was the first study to characterize both bacterial and fungal communities of winter OSR roots, rhizosphere and bulk soil simultaneously (figure 10). Proteobacteria and Actinobacteria were the most dominant bacterial phyla in the three compartments, while Ascomycota and Basidiomycota were the two dominant

fungus phyla in the bulk soil and rhizosphere. The root compartment was associated with Chytridiomycota and a higher abundance of unidentified phyla. Other plant species of the Brassicaceae family showed similar results but for bacteria only: Proteobacteria, Actinobacteria and Bacteroidetes were the most abundant bacterial phyla in *Boechera stricta* roots (Wagner *et al.*, 2016) while Ascomycota and Basidiomycota were the most abundant fungal phyla in *Microthlaspi* roots (Glynou *et al.*, 2018). Regarding plants associated to arbuscular mycorrhizal fungi, barley roots were mainly colonized by Ascomycota and Basidiomycota (Wilkinson *et al.*, 2019).

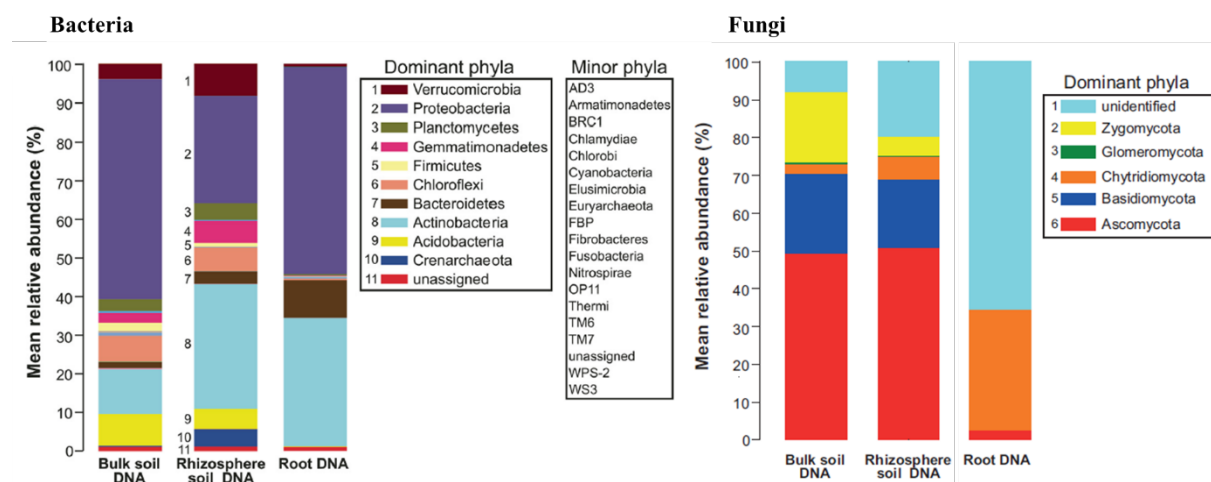


Figure 10 Mean relative abundances of different bacterial and fungal phyla in bulk soil, rhizosphere soil and root DNA (modified from Gkarmiri *et al.*, 2017). Colors for dominant phyla that are easily distinguishable are shown in a separate legend, supplemented with a numerical key. Minor phyla are simply listed.

4.2. The insect holobiont: the cabbage root fly (CRF, *Delia radicum*)

4.2.1. Taxonomy and identification

The cabbage root fly (“CRF”) *Delia radicum* (Linnaeus) is a fly from the Anthomyidae family (Insecta, Diptera), and it carries many names in the literature: *Hylemya brassicae* (Bouché), *Hylemyia brassicae*, *Erioischa brassicae* (Finch, 1989; Savage *et al.*, 2016). Several species are associated to the *Delia* genus: *D. antiqua*, (Meigen), *D. floralis* (Fallén), *D. florilega* (Zetterstedt), *D. planipalpis* (Stein), *D. platura* (Meigen) and *D. radicum*, which can be distinguished using identification keys at different development stages (Darvas and Szappanos, 2003; Savage *et al.*, 2016).

This fly is a holometabolous insect since it undergoes a complete metamorphosis, starting from the eggs, followed by three larval instars, with the latter instar transforming into a pupa (figure 11A). This transformation separates the immature and juvenile stages from the

final mature and adult stage. Adult individuals can be relatively easily distinguished between males and females. Generally, the male has a thin abdomen and narrow gap between its eyes, while the female has a bigger abdomen and larger gap between its eyes.

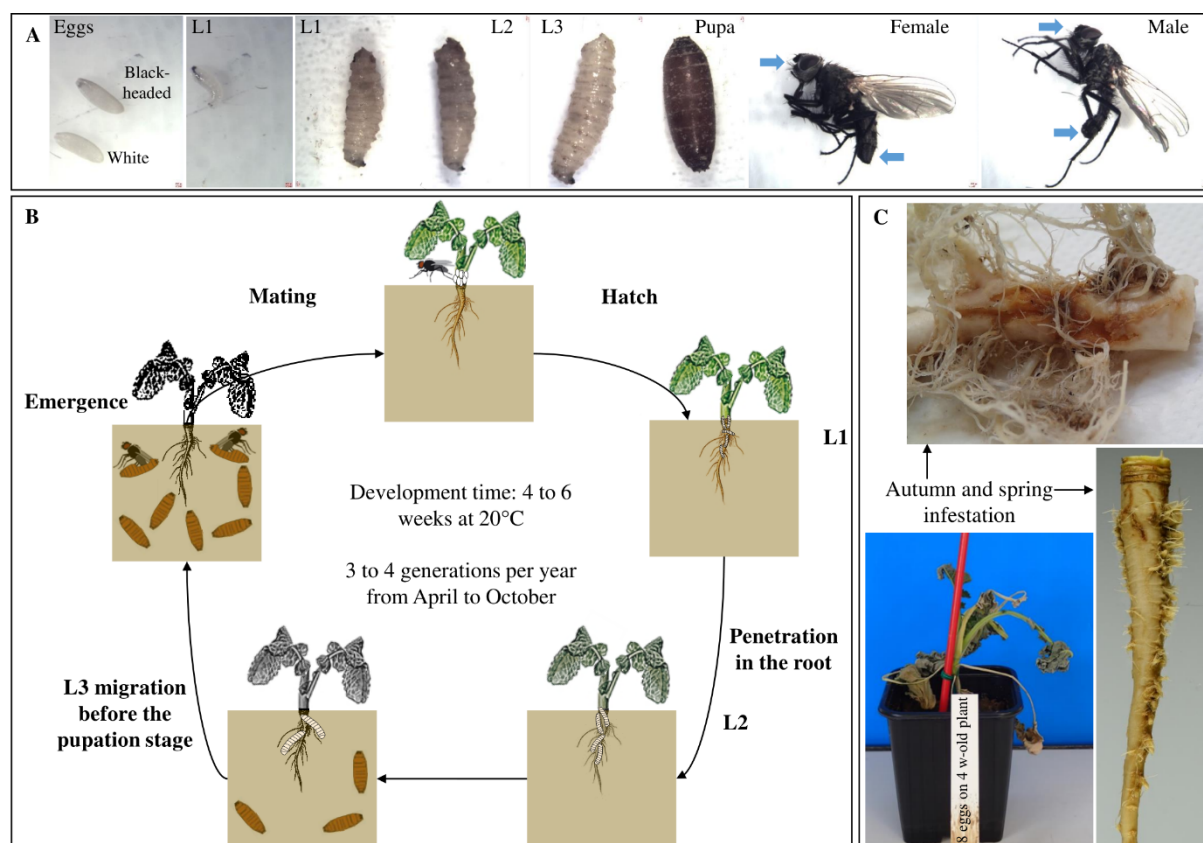


Figure 11 Identification card of the cabbage root fly, *Delia radicum*. (A) Development stages of the cabbage root fly. Blue arrows indicate morphological differences between males and females regarding the abdomen size (♀: round, ♂: thin) and the gap between eyes (♀: large, ♂: narrow). (B) Biological cycle of *D. radicum* on winter oilseed rape. (C) Root damages on winter oilseed rape plants caused by *D. radicum*.

4.2.2. Host plants and development

In field conditions, the CRF is a specialist of the Brassicaceae family, that can develop on different host plants either wild or cultivated crops like radish, turnip, rutabaga, cabbage, cauliflower or oilseed rape (Finch and Ackley, 1977). Following figure 11B, *D. radicum* biological cycle starts with the female prospecting, in order to select the host plant where to oviposit (i.e. action of laying eggs using a specialized organ, ovipositor) on the stem base (i.e. crown). A host plant will be chosen by the female based on its recognition of signals emitted by the plants (Košťál, 1991; Košťál *et al.*, 2000; Gouinguéné *et al.*, 2005; Gouinguéné and Städler, 2005; Gouinguéné *et al.*, 2006; Gouinguéné and Städler, 2006b). Eggs hatch within a few days and the three larval instars feed by tunneling into the roots for 2–3 weeks, before

pupating in the nearby soil. Adult emergence occurs about a month after oviposition and can live up to 45 days when supplied with food and water (Finch and Coaker, 1969).

In the laboratory, the flies live in rearing cages (Bug Dorm-4 Insect Rearing Cage, 47.5 x 47.5 x 47.5cm) in a climatic room (16:8 LD, $21 \pm 2^{\circ}\text{C}$; $60\% \pm 10\%$ RH). They get water from wet cotton and are fed on a mixture composed of sugar, dried milk and yeast (ratio 1:1:1). Flies reproduce in these cages and females lay their eggs on pieces of rutabaga roots (*Brassica napus* subsp. *rapifera*). Then the eggs are removed carefully using a pencil so they can be placed on a whole new rutabaga to avoid overcrowding. A bag is placed on top of the pot containing the latter rutabaga resting on a layer of sand. After emergence, flies are going back into the rearing cages.

4.2.3. Damages and distribution

The development stage of *D. radicum* the most harmful to the plant is the larval stage when the maggots feed on the roots. They form galleries inside the roots, as well as some that are visible on the outside (figure 11C). Such damages are also accompanied with root decay, which pathogens use as an opportunity to invade the roots. External symptoms of larval herbivory can be observed through the leaf yellowing up to their withering. In case of strong infestation, larvae can sever the plant xylem vessels, which transport water from the roots to the stem and leaves, provoking the plant desiccation, and sometimes death (figure 5C).

Delia radicum has a range of dispersion that seems to be within a 2-3 km radius of the site of infestation with the females going further than males (Finch and Skinner, 1975). This species can be found developing in temperate zone of the Holarctic region (table 2), such as North America including Canada, or Europe (Finch, 1989; Lamb, 1989). Moreover, the CRF is a major insect pest of canola in Canada, and Dosdall *et al.* (2012) observed that damages caused *D. radicum* larvae have increased during their study conducted between 2008 and 2010.

Table 2 Occurrence of the cabbage root fly in Europe and in the USA reported in publications between 2007 and 2014.

Area	Sub-area	Date	Plant species	Number of eggs [†] (mean ± SE)	Number of pupae [‡] (mean ± SE)	Study
Denmark	Årslev	July 2007	White cabbage	42.6 ± 13.8 [*]		Meyling <i>et al.</i> (2013)
		November 2007			8 [*]	
		July 2008		27.3 ± 15.3 [*]		
		November 2008			3 [*]	
		July 2009		16.3 ± 3.2 [*]		
		November 2009			5 [*]	
France	Brittany (St Pol de Léon)	April-June 2010	Broccoli	28.8 ± 3.1	13.4 ± 1.4	Josso <i>et al.</i> (2013)
	Brittany (Tréguier)	April-June 2010		91.9 ± 15.6	13.7 ± 1.9	
	Brittany (Le Rheu)	April-May 2014	Broccoli	8.9 ± 0.4	12.9 ± 0.8	Lamy <i>et al.</i> (2018)
		April-May 2014	Chinese cabbage	6.9 ± 0.5	21.6 ± 2.3	
USA	California (Chualar)	Summer 2013	Broccoli	Approx. 286 [#]	–	Joseph and Martinez (2014)
	California (Soledad)	Fall 2013		Approx. 344 [#]	–	

[†]Number of eggs per plant and per week. [‡]Number of pupae per plant and per week. ^{*}Estimation from graphics of conventional farming. [#]Sum on 30 basal-soil and 100 broccoli root samples.

4.2.4. Microbial ecology of *D. radicum*

Several studies have characterized *D. radicum* bacterial communities at different stages. Lukwinski *et al.* (2006) has detected cultivable bacteria at the egg stages but also identified several bacterial species (*Pseudomonas*, *Erwinia* and *Serratia*) from the Enterobacteriaceae family which are part of the γ -Proteobacteria class, in the larval gut. By assessing the biochemical activities in the gut, these authors found out that several groups of bacteria were associated with hydrolysis activity. Such results were confirmed later by Welte *et al.* (2016), detecting 11 strains of γ -Proteobacteria (figure 12), which possessed plasmids containing a *saxA* gene responsible for the degradation of an isothiocyanate, a toxic volatile organic compound originating from the hydrolysis of glucosinolates by myrosinase enzymes. Regarding the adult stage, it was described by Bili *et al.* (2016) in a context of parasitoid-host interactions, where two *D. radicum* populations (western and eastern Brittany) shared 4 bacterial genera (*Wolbachia*, *Deftia*, *Ralstonia* and *Spiroplasma*) with the most abundant being *Wolbachia*. Both *Wolbachia* and *Spiroplasma* are known to be heritable. *Enterococcus*, *Asaia* and *Gluconacetobacter* genera were exclusively identified in the eastern population while *Bacillus* was only detected in the western one. The study of Bili *et al.* (2016) showed that even though there is a strong trophic interaction between the CRF and its parasitoids, only a few bacterial genera were shared.

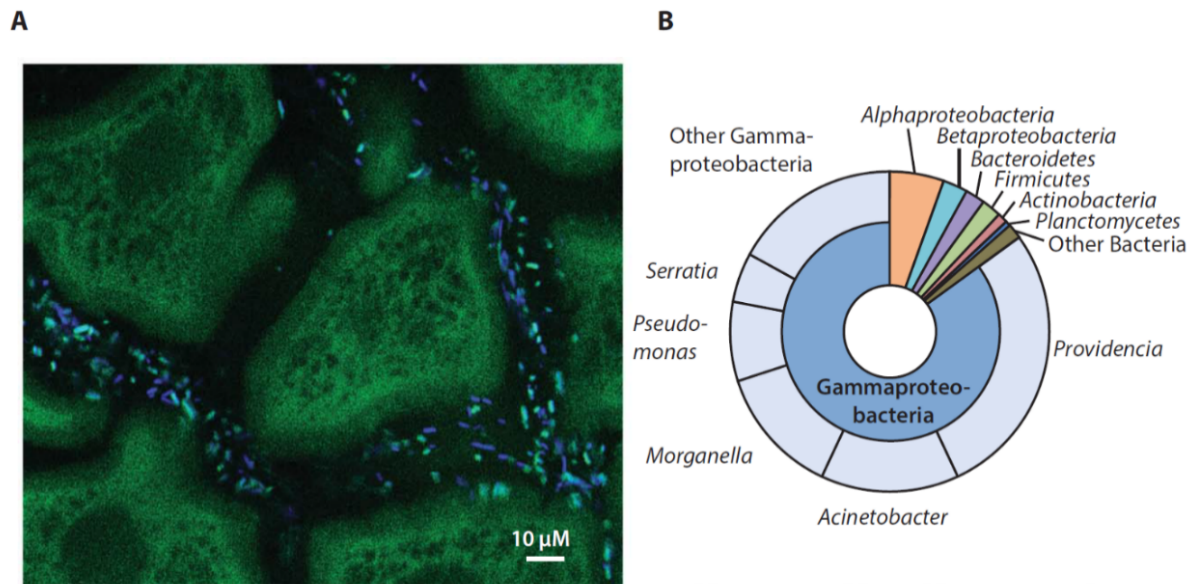


Figure 12 Visualization of the gut microbiota of *D. radicum* larvae (Welte *et al.*, 2016). (A) Micrograph of the *D. radicum* midgut after fluorescence in situ hybridization. The probes used were directed against Bacteria (probe Eub-mix, cy-5, blue label) and most Gammaproteobacteria (probe GAM42A, fluos, green label). Gammaproteobacteria appear light blue, whereas Bacteria not hybridizing with the GAM42A probe appear in dark blue. *Delia radicum* gut epithelial cells appear green by autofluorescence. (B) Pie chart representation of 16S rRNA gene abundance of different bacterial genera and phyla as determined by metagenome sequencing.

5. Objectives and organization of the manuscript

During this PhD, I have studied the tripartite interactions between two holobionts: the winter oilseed rape (*B. napus*), the cabbage root fly (*D. radicum*) and their associated microbiota in order to assess the role of soil microorganisms and root herbivory within the plant-insect-microorganism interactions and to describe insect bacterial communities.

Following this general introduction (chapter I), the manuscript contains three chapters presenting results from different experiments to study the tripartite interactions between *B. napus*, *D. radicum* and their associated microbial communities (figure 13).

In chapter II, we aimed at determining the effect of soil microbiota diversity on *B. napus*-*D. radicum* interaction. To do so, we experimentally manipulated the soil microbial diversity, using a dilution to extinction approach to obtain a microbial diversity gradient that we tested on the fly development (paper 1: Lachaise *et al.*, 2017) and behavior (short note 1) and investigated whether plant chemistry could explain impact on these life history traits.

In chapter III, we assessed the influence of *D. radicum* herbivory on plant chemistry and microbial communities. For that purpose, plant microbiota dynamic was followed through the insect development under both healthy and herbivory conditions. In order to understand herbivory-induced microbial changes, root chemistry was analyzed in parallel of root and rhizosphere microbial communities. The finality was to identify potential relationships between plant chemical and microbial modifications due to herbivory (paper 2: Ourry *et al.*, 2018).

In chapter IV, we aimed at characterizing the microbiota of *D. radicum* and its acquisition. In a first part, we assessed the effects of an antibiotic on the fly microbiota and its recovery after two generations of successive antibiotic treatment (paper 3). In a second part, microbial vertical transmission was investigated by comparing different fly generations, while horizontal transmission or the contribution of the environment into shaping insect microbial communities was evaluated by comparing microbiota from different development stages of the fly to the microbiota of the plant compartment that the fly interacts with (paper 4).

Finally, a general discussion (chapter V) closes this manuscript by summing up the main results and presenting the perspectives of this work.

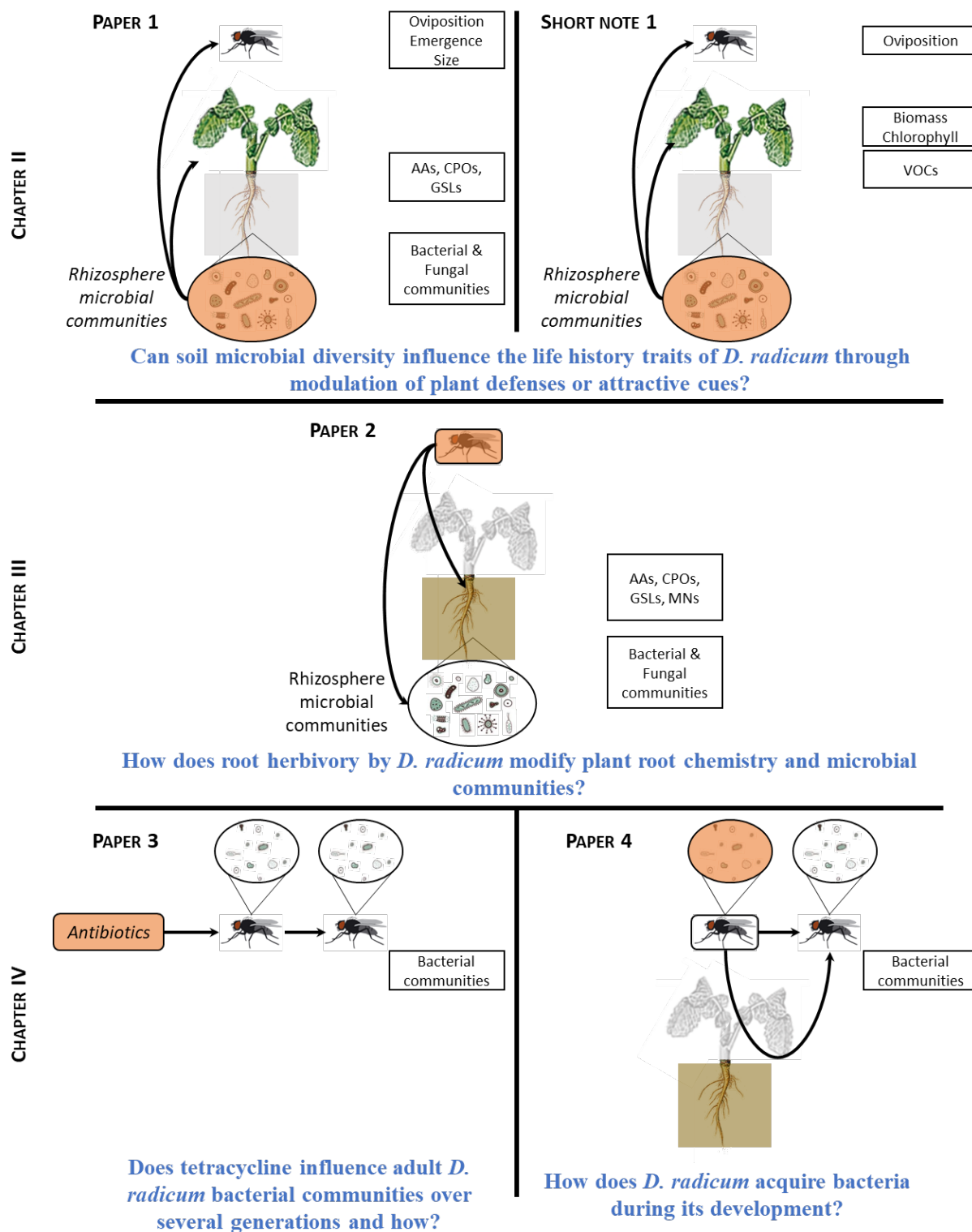
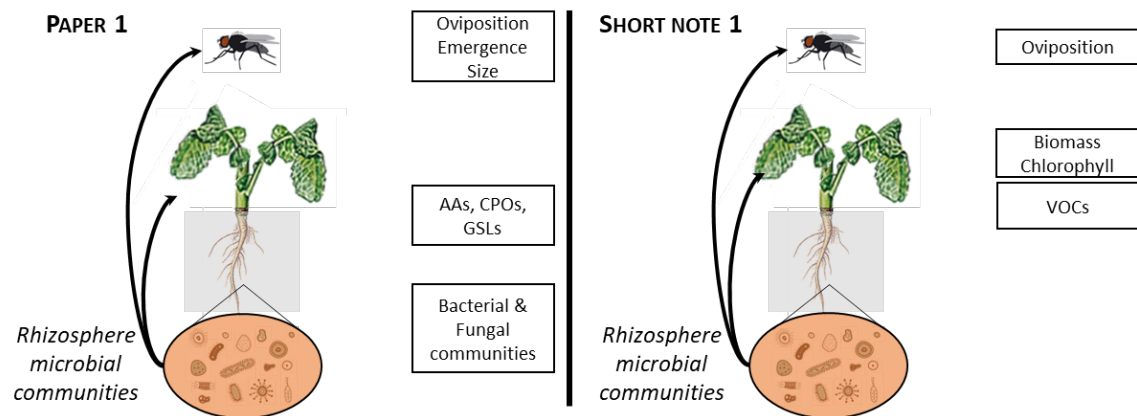


Figure 13 Overview of the manuscript organization to study the quadripartite interaction between *B. napus*, *D. radicum* and their associated microbial communities, with the research questions written in blue. The studied factors are colored in orange and their studied effects are represented by arrows.
AAs: Amino Acids; CPO: Carbohydrate, Polyol and Organic Acids; GSLs: Glucosinolates; VOCs: Volatile Organic Compounds; MNs: Macro- and micronutrients.



CHAPTER II

SOIL MICROBIAL COMMUNITIES, MODULATORS OF PLANT CHEMISTRY AND INSECT LIFE HISTORY TRAITS?



Can soil microbial diversity influence the life history traits of *D. radicum* through modulation of plant defenses or attractive cues?

CHAPTER II: SOIL MICROBIAL COMMUNITIES, MODULATORS OF PLANT CHEMISTRY AND INSECT LIFE HISTORY TRAITS?

Presentation of the chapter

Context

Many studies have demonstrated the plant abilities to produced chemical defenses against herbivorous insects. Primary and secondary plant metabolites play a pivotal role in insect behavior and development but also in microbial recruitment and interactions. Moreover, some of these soil microorganisms have been recognized as being key players that help maintaining the plant health, for example by improving the plant nutrition or resistance to pathogens. Such effects from soil microorganisms have also shown to be beneficial for plants when being attacked by herbivorous insects. Indeed, several of these microorganisms are able to induce an increase of plant defenses as well as negatively affect the insect development. Until recently, most of these studies have been conducted on microorganisms at the species or strain level, without considering microorganisms interacting with each other on a community scale.

There is a lack of knowledge regarding the implication of soil microbial communities, and their diversity in plant-insect interactions and their potential impact on belowground herbivorous insects through modifications of their host plants.

Approach

Our aim was to determine whether soil microbial diversity affects a root herbivore development and behavior through plant chemistry modulations, using oilseed rape (*Brassica napus*) and the cabbage root fly (*Delia radicum*).

To do so, we used an experimental approach: the dilution to extinction method, which consists of diluting a soil inoculum in order to obtain a decreasing gradient of microbial diversity that serves to reinoculate a sterile soil matrix. After reinoculation, the soils were incubated to reach the biological capacity of the soil (maximum abundance of the microbiota). Three soils of different microbial diversity (i.e. high, medium and low) were obtained and used to grow *B. napus*. Initial soil microbial diversities were analyzed with Illumina MiSeq sequencing and 16S and 18S primers, to assess bacterial and fungal communities respectively just before sowing. After several weeks of plant cultivation, two experiments were performed.

First, *D. radicum* emergence rate (number of emerged adults reported to the number of eggs deposited on the plant crown), size (i.e. hind tibia length), oviposition (i.e. number of eggs laid on the plant crown when female flies were in presence of only one host plant) were measured in parallel with quantification of *B. napus* amino acids, sugars and glucosinolates in the roots and leaves (paper 1).

Second, *D. radicum* oviposition was measured in a choice experiment (i.e. female flies in presence of several host plants) in parallel with the assessment of plant leaf and root dried biomasses and chlorophyll content as well as the detection of leaf *B. napus* volatile organic compounds (VOCs) using an olfactometer(short note 1).

Main results

We have demonstrated that our experimental approach successfully led to obtaining soils that differed only from their microbial diversity, as the sterile matrix used for reinoculation was the same for all dilutions, which implied that these soils had similar physicochemical properties. Different bacterial and fungal communities were obtained, forming a decreasing gradient of microbial diversities.

Such communities impacted *D. radicum* emergence rate, higher on medium level of soil microbial diversity, but not its size nor its oviposition in a non choice experiment. These results could not be linked to chemical changes as only the infestation condition, at the beginning of herbivory and at the emergence, influenced the plant chemistry by decreasing root and leaf amino acid and glucosinolate contents, as well as root sugar content.

The oviposition not influenced by a change in microbial diversity could be due to the setting that was a non choice experiment, where the female flies might still lay their eggs despite the plant being not the better host but the only available one. Indeed, when offered plants that grew on different soil microbial diversities, females displayed preferences that varied as the plant aged: on low and medium diversities when plants were 4 and 6 weeks old respectively. These choices could not be explained by the plant biomasses or the VOC composition, as they were not influenced by the soil microbial diversities. However, chlorophyll content tended to be slightly higher (i.e. greener plants) on low diversity than medium in 4 week-old plants, which could have attracted the flies, while there was no difference in 6 week-old plants.

Our study highlighted the potential effects of soil microbial communities on *D. radicum* life history traits but we are still unable to identify the underlying mechanisms responsible for these changes.

Limits

The two studies were conducted separately with different inoculum preparation and one year apart. As the soil diversity was assessed only in the first study, it raises the question of the stability of the microbial inoculum over time.

The first study assessed metabolite contents of both roots and leaves at two different times: one day after larval infestation and at adult emergence. These two times correspond to the start and end of plant-insect interaction, respectively when larvae would be on the root surface and when flies end their pupation in the soil that lasts for one to two weeks with no contact with the plant during this period. However, plant-insect interaction is the strongest when larvae feed by tunneling inside the roots two weeks after hatching, which is when major chemical changes are consequently expected. Moreover, if metabolites are submitted to diurnal oscillations, it is not surprising that they would also vary over the plant development. In the absence of comparison between metabolites of healthy and infested plants at adult emergence, it is not possible to distinguish metabolite variations due to the plant development from the ones due to *D. radicum* herbivory, when in addition plants are interacting with different soil microbial diversities.

The second study used different sets of plants for the oviposition choice experiment, which also included biomass and chlorophyll measurements, and for the leaf VOC detection. Originally, these two sets were associated to two different experiments, which were conducted over a year apart. As each set of plants was mostly grown under natural photoperiod and VOC production tends to be as sensitive as metabolites, conclusions about the causal relationship between the two datasets should be made with precaution.

As previously mentioned, plant chemistry was assessed in our studies but root VOCs were not measured. However, microorganisms are also known to produce chemical compounds like metabolites and VOCs and our experiments were not designed to untangle chemicals produced by the plant from the ones produced by microorganisms.

Perspectives


To go further and determine the drivers responsible for the fly phenotype changes, other plant defenses well-documented in the literature should be measured like other chemical compounds or root physical defenses and root VOCs should also be taken into consideration. In order to better characterize metabolite changes during plant-insect interaction, key phases that follow the insect development stages should be retained so that the dynamics of plant-insect interaction could be described.

Publication status

Paper 1, entitled “Can soil microbial diversity influence plant metabolites and life history traits of a rhizophagous insect? A demonstration in oilseed rape”, was published as a scientific paper in the 2017 special issue of *Insect Science*: “Plant-insect-microbe interactions”. Short note 1 has not been submitted yet.

ORIGINAL ARTICLE

Can soil microbial diversity influence plant metabolites and life history traits of a rhizophagous insect? A demonstration in oilseed rape

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Abstract Interactions between plants and phytophagous insects play an important part in shaping the biochemical composition of plants. Reciprocally plant metabolites can influence major life history traits in these insects and largely contribute to their fitness. Plant rhizospheric microorganisms are an important biotic factor modulating plant metabolites and adaptation to stress. While plant–insects or plant–microorganisms interactions and their consequences on the plant metabolite signature are well-documented, the impact of soil microbial communities on plant defenses against phytophagous insects remains poorly known. In this study, we used oilseed rape (*Brassica napus*) and the cabbage root fly (*Delia radicum*) as biological models to tackle this question. Even though *D. radicum* is a belowground herbivore as a larva, its adult life history traits depend on aboveground signals. We therefore tested whether soil microbial diversity influenced emergence rate and fitness but also fly oviposition behavior, and tried to link possible effects to modifications in leaf and root metabolites. Through a removal-recolonization experiment, 3 soil microbial modalities (“high,” “medium,” “low”) were established and assessed through amplicon sequencing of 16S and 18S ribosomal RNA genes. The “medium” modality in the rhizosphere significantly improved insect development traits. Plant–microorganism interactions were marginally associated to modulations of root metabolites profiles, which could partly explain these results. We highlighted the potential role of plant–microbial interaction in plant defenses against *Delia radicum*. Rhizospheric microbial communities must be taken into account when analyzing plant defenses against herbivores, being either below or aboveground.

Key words *Brassica napus*; *Delia radicum*; life history traits; plant primary and secondary metabolites; rhizophagous pest; soil microbial diversity

Introduction

Interactions between plants and phytophagous insects play an important part in shaping the biochemical composition

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of plants. Primary and secondary metabolites can influence fitness in phytophagous insects through several developmental or behavioral traits (Nishida, 2014). Microorganisms are also known to have a significant influence on life history traits of insects. From obligate symbionts (e.g., *Buchnera*), to necrotrophic pathogens (e.g., *Cordyceps*), endosymbiotic microbiota effects on nutrition, immunity, development and reproduction have been documented in many insect species (Feldhaar, 2011). Most studies focused on the insect microbiota, neglecting interactions between the plant and the insect and in particular, the potential role of the plant rhizosphere microbiota. This plant microbiota can influence phytophagous insects directly, for instance via endophytic and epiphytic activities of entomopathogens (Klieber & Reineke, 2016), but also indirectly through mutualistic interactions with plant roots involving fungi or bacteria (e.g., mycorrhiza and Plant Growth Promoting Rhizobacteria). These symbiotic associations can modulate plant metabolites production, shifting its nutritional value for phytophagous insects (Pineda et al., 2010). Some bacterial and fungal strains are also able to prime plant defense against herbivores via induced systemic resistance (ISR), which corresponds to the plant immune response against pathogens. For instance, ISR has been shown to boost defensive compounds at the transcriptional level, impairing insect development (Pangesti et al., 2015). However, induced defenses have mostly been studied using cultivation approaches and a few strains of microorganisms, while the effects of whole microbial communities are poorly known. To date, only 1 study measured the effect of rhizospheric microbial diversity on plant–herbivores interactions and showed that diversity levels differentially modulate leaf plant metabolites after infestation by aphids but also the size of insects developing on these leaves (Hol et al., 2010). However, these authors studied the effect of soil microbial dilutions on leaf (and not root) metabolites and on an aboveground insect, which does not interact directly with the soil. So far, no study has focused on the effects of soil microbial diversity on above and belowground plant metabolites and on life-history traits of a belowground insect.

Here, we tackled this question using oilseed rape (*Brassica napus*) and the cabbage root fly (*Delia radicum*) as biological models. Even though *D. radicum* is a belowground herbivore as a larva, its adult life history traits depend on aboveground signals. This fly is a specialist rhizophagous herbivore of brassicaceous plants. Females select host plants based on their aboveground physical and chemical characteristics and lay their eggs at the base of plant stems (Gouinguéné & Städler, 2005). Eggs hatch within a few days and the 3 larval instars feed by tunneling into the roots for 2–3 weeks, before pupating in the nearby

soil. Adult emergence occurs about a month after oviposition. This fly can cause severe damage on plant roots and is considered an important pest on several brassicaceous crops, including oilseed rape (Ahuja et al., 2010).

We evaluated the effect of rhizospheric microbial diversity on (i) primary and secondary metabolites of roots and leaves in healthy and infested plants and (ii) insect oviposition and development.

Materials and methods

Microbial communities

Soil preparation: obtaining different microbial diversity levels The soil used in our experiments was collected in November 2014 from the layer –10 and –30 cm of a cultivated field in Brittany (La Gruche, Le Rheu, France, 48°08'44"N, 01°47'97"W). The main physico-chemical characteristics are shown in supplementary data (S1). In order to remove the macrofauna, soil was homogenized, ground and sieved at 4 mm before being mixed up with silica sand (2/3 soil, 1/3 sand). A part of this mixture was sterilized by gamma radiation (35 kGy) and stabilized for 2 months before inoculation. The unsterilized remnant soil was ground, sieved at 2 mm, with no silica added and was used by suspending 100 g of dry unsterilized soil into 1 L of deionized water. The mixture was either undiluted (10^0), diluted at 10^{-3} or 10^{-6} , and solutions were agitated between each dilution step. Each dilution process was repeated 3 times to obtain 3 biological replicates (referred to as soil biological replicates). Bags containing 2.5 kg of sterilized soil were inoculated with 300 mL of each dilution, creating a microbial diversity gradient (respectively “high,” “medium,” and “low” modality) or soil microbial modalities, and incubated in the dark at 18°C and 50% humidity for 49 d. During this period, the bags of soil were opened and homogenized every week to facilitate microbial respiration and recolonization. A microbiological follow up, based on the colony forming units method was performed during the incubation period to insure that soil reached optimal fungi and bacteria densities and similar abundances between the 3 soil microbial modalities.

Molecular and bioinformatical analyses Before each sowing, the soil was sampled to assay the 3 microbial modalities ($N = 15$ samples per modality, unevenly distributed among plant sowing). DNA was extracted from 2 g of dry soil, then purified by PVPP column and by GeneClean (Plassart et al., 2012). PCR amplification and sequencing were performed at the GenoScreen (Lille, France) using the Illumina Miseq platform to a

2 × 250 bases paired-end version. PCR was realized using primers: F479 (5'-CAGCMGCGYGCNGTAANAC-3') and R888 (5'-CCGYCAATTCMTTTRAGT-3'), and also FR1 (5'-AICCATTCATTCGGTAIT-3') and FF390 (5'-CGATAACGAACGAGACCT-3') to amplify 16S and 18S rDNA genes, respectively. To manage mismatch between reads in the overlap region, we used bases trimming at Q30 with PRINSeq, trimming of specific primers with Cutadapt, assembly with FLASH starting with trimmed read1 and 2, a minimum of 30 bases overlapping was required with a 97% homology between reads. Bioinformatical analyses and diversity indices calculation followed (Terrat *et al.*, 2012). The total number of Operational Taxonomic Units (OTUs), and the Shannon and Evenness indices were used as indicators of microbial richness, diversity and evenness, respectively. Raw data sets were deposited on the European Nucleotide Archive (ENA) database system under the accession number (PRJEB20587). Normalization step by the methods of relative abundance (1000 × counts of gene X/total number of reads) and the trimmed mean of *M* values was performed before analyzing the diversity and structure of bacterial and fungal communities respectively.

Plants

Growing conditions Seeds of *Brassica napus* L. cultivar (subsp. *oleifera* cv. Tenor) were sown in individual pots filled with 1 of the 3 soil microbial modalities and resting on a layer of pozzolan. Plants were put in climatic chambers (L 16 h : D 8 h, 20 °C/18 °C) during 1 week. Then, they were placed in a greenhouse under natural late 2015 winter photoperiod (mean temp. 14.7 °C, min/max 5.8/22.1 °C). Watering was performed by subirrigation every 2–3 d with Hoagland 1X solution (Hoagland & Arnon, 1950) supplemented with iron (Fe EDTA Na, 27 nmol/L). After the beginning of the experiments, pots were moved back to climatic chambers. Plants used for oviposition and development experiments were 6 weeks old. Three treatments were applied in the development experiment which plants were then used for metabolic profiling. Uninfested plants and 1 day after infestation (“dai”) plants were 6–7 weeks old while plants harvested after fly emergence were 11 weeks old and had been infested for the last 5 weeks (Fig. 1). These 3 treatments were referred to as “healthy,” “1 dai,” and “35 dai” plants or treatments. For the technical feasibility of the experiments, 3 sowings were performed at 1-week interval. Nine plants per soil microbial modality and coming unevenly from 2 sowings were analyzed for metabolites assessment for both “healthy” and “1 dai” treatments while 9 plants per soil microbial modality com-

ing from the third sowing were used for the “35 dai” treatment.

Biochemical analyses Root metabolites were quantified for the 3 treatments: “healthy,” “1 dai” and “35 dai” plants while leaf metabolites were analyzed only for “healthy” and “1 dai” treatments. After collection, root and leaf samples (leaves no. 6–7–8 were mixed) were plunged into liquid nitrogen and kept at –80 °C until they were freeze-dried and ground. The analyzed compounds were amino and organic acids, sugars, and glucosinolates (GSLs).

Amino acids (AAs), sugars and organic acids (SOAs) were extracted and derived according to Gravot *et al.* (2010), with the following modifications: (i) samples were agitated for 10 min after adding chloroform, (ii) 5 µL of extract were used to the AA derivation, and (iii) SOA derivation was realized online. This derivation required 50 µL of a pyridin–methoxyamin solution added to the dried samples, agitated briefly and incubated for 90 min at 40°C before adding 50 µL of MSTFA (N-methyl-trimethylsilyltrifluoroacetamide) and a second agitation-incubation for 30 min at 40°C. Metabolites retention times were compared to an external standard. AAs were analyzed by liquid chromatography (UPLC, Waters, Milford, MA, USA) and quantified with BABA as an internal standard. SOAs were analyzed using gas chromatography (GC, Thermo Fisher Scientific, Waltham, MA, USA) and quantified with an adonitol internal standard. The GC was composed of a FID, a SSL injector, and a TG-5MS column.

Glucosinolates (GSLs) were extracted according to Hervé *et al.* (2014), with the following modifications: (i) the extraction required 10 mg of dry matter, (ii) samples were centrifuged 5 min at 12 000 × *g* and 15°C after the ultrasonic bath, and (iii) leaf samples were filtrated. Samples were injected and analyzed by liquid chromatography coupled with a mass spectrometry (UPLC-PDA-MS TQD Aquity, Waters) and quantified with a calibration range of an external standard composed of glucoerucin, gluconasturtiin, and glucobrassicin (dilutions: 3, 5, 10, and 50). They were respectively used to quantify aliphatic, aromatic and indolyl GSLs.

Insects

Rearing The cabbage root flies (“CRF,” *Delia radicum*) used in our experiment came from a rearing (University of Rennes 1, Brittany, France) started with individuals collected in cabbage fields in 2014 (Le Rheu, Bretagne, France, 48°07'16"N, 01°47'41"W). Rearing conditions are adapted from Neveu Bernard-Griffiths

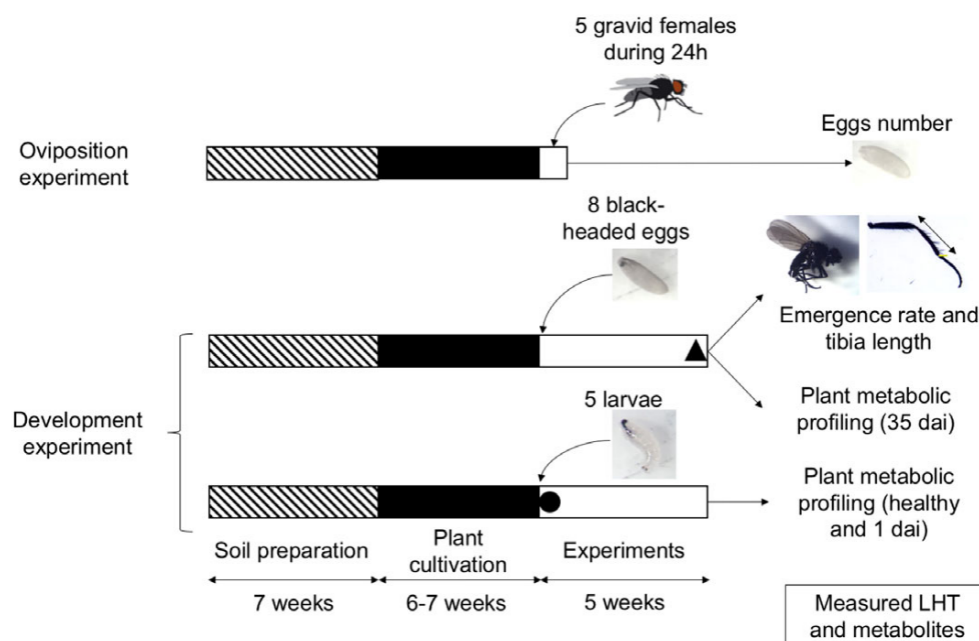


Fig. 1 Experimental designs to evaluate life history traits (LHT) of *Delia radicum* and plant metabolites. Seven weeks of soil preparation (striped bars), which produced 3 microbial modalities (“high,” “medium,” and “low”) and 6–7 weeks of cultivation (black bars) were required to obtain plants used in our experiments (white bars). For the oviposition experiment, 5 gravid females (10-d-old) were enclosed with a plant during 24 h before being removed to count the number of eggs laid. For the development experiment, 8 black-headed eggs (i.e., 3-d-old and ready to hatch) were placed on the plant stem base. Four weeks later, corresponding to 35 d after infestation (dai), emergence rate and tibia length were measured while root metabolites content were analyzed. Metabolites from the “healthy” and “1 dai” treatments were quantified 24 h after those plants were infested with 5 larvae of 10-d-old (i.e., “1 dai” treatment). Plant collection for metabolic profiling from “healthy” and “1 dai” treatments is symbolized by a black circle while it is represented by a triangle for the “35 dai” treatment. $N = 81$ plants per experiment with 27 plants per microbial modality for oviposition and development experiments (i.e., fly LHT measurement). $N = 27$ plants per soil microbial modality for “healthy” and “1 dai” treatment but only 9 per soil microbial modality for the “35 dai” treatment regarding metabolites analyses.

(1998) and took place in controlled conditions (L 16 h : D 8 h, $21 \pm 2^\circ\text{C}$; RH $60\% \pm 10\%$). Oviposition experiments used 10-d-old gravid females fed on milk powder, sugar and nutritional yeast (1 : 1 : 1) until the beginning of experiments. For the developmental experiment, we used eggs at the “black-head” stage (i.e., about to hatch) obtained by letting females lay eggs on rutabaga roots (*Brassica napus* subsp. *rapifera*) for 24 h and letting eggs develop for 3 d before collection. Plants from the “1 dai” and “35 dai” treatments were infested by five 10-d-old larvae and 8 “black-headed” eggs, respectively (Fig. 1).

Life history traits experiments Oviposition—Five 10-d-old gravid females were enclosed with a potted plant using a transparent plastic bag (30×50 cm). Eggs were counted after 24 h ($N = 27$ plants per soil microbial modality).

Development and fitness—“Black-headed” eggs as 10-d-old larvae were deposited on the stem base of each

potted plant before the pot was enclosed and placed in a climatic chamber. After 5 weeks of infestation, emerging flies, which came from the “35 dai” treatment, were collected and counted every morning during 4 d since the first insect emerged. Emergence rate was estimated as the ratio between the number of emerged flies and the 8 eggs deposited. To assess fly fitness, the length of the hind tibia was measured using a CMEX-5000 camera fitted to a dissecting microscope. Oviposition, emergence and tibia length experiments were replicated 27 times per soil microbial modality ($N = 9$ plants per 3 sowings).

Statistical analyses

Analyses were realized with the R software (R Core Team, 2016), associated to the “RVAideMemoire” package (Hervé, 2016a, b).

Microbial communities Sequencing data were filtered, normalized and analyzed according to the script provided by Bulgarelli *et al.* (2012). From there, richness (number of observed OTUs per sample), the Shannon and Pielou indices were calculated for bacteria and fungi. The Shannon index corresponds to alpha-diversity, defined as the mean species diversity in a habitat at a local scale, and was calculated using the “vegan” package and the “diversity” function (Oksanen *et al.*, 2016). The Pielou index represents evenness, the closeness between abundances of each species in a habitat. A one-way ANOVA was used to measure the effect of soil microbial modality (“high,” “medium,” and “low”) on these 3 indices. If significant and if the normality of residuals was respected (graphical assessment via QQplots), pairwise comparisons were performed with a Tukey’s test.

To analyze beta-diversity or structural differences between microbial communities, sequencing data were filtered and normalized using the “edgeR” package (Robinson *et al.*, 2010; Anders *et al.*, 2013). This dataset was then used in 2 different analyses. Data were first reduced and centered to create a new matrix based on the Bray–Curtis dissimilarity index, in order to realize a Principal Coordinate Analysis (PCoA) and to carry out permutation tests to detect differences between community structures. Genewise negative binomial Generalized Linear Models were then built to recover genera differences between microbial modalities for both bacteria and fungi.

Plants Metabolites were retained for statistical analyses when they exceeded the mean content threshold of 1 nmol/mg. Linear mixed models following log transformation were used to analyze total AA, total SOA, and total GSL content for both roots and leaves, with treatment (“healthy,” “1 dai,” “35 dai” for roots, and “healthy,” “1 dai” for leaves), soil microbial modality, soil biological replicates as fixed variables and sowing as a random variable. The normality of residuals was checked before performing a Wald test on the model. *P*-values obtained were then corrected with the False Discovery Rate method. When significant, pairwise comparisons were performed on least square means of the studied variables.

Insects Oviposition, development, and fitness—Effect of soil microbial modality on oviposition behavior and adult emergence was evaluated using a generalized linear mixed model on number of eggs and emergence rate using the Negative Binomial and Binomial families respectively. Soil microbial modalities and soil biological replicates were used as fixed variables with sowing as random variable for both analyses. Emergence analysis

required to use plants as random variables in order to suppress data overdispersion. Model validity was checked with the “plotresid” function before performing a Wald test on the model. When it was significant, pairwise comparisons between microbial modality were performed on least square means of the studied variables. A linear mixed model was applied to analyze the hind tibia length with soil microbial modalities and biological replicates as fixed variables and sowing and plants as random variables.

Results

Microbial communities

Richness of bacterial and fungal communities, represented by the number of observed OTUs, significantly decreased from the highest to the lowest soil microbial modality, with 350, 320, and 290 OTUs in bacterial communities (ANOVA: $F = 131.7$, $df = 2$, $P < 0.001$, Fig. 2A) and 90, 80, and 70 OTUs in fungal communities (ANOVA: $F = 17.3$, $df = 2$, $P < 0.001$, Fig. 2B). The same decline was observed for alpha-diversity (Shannon index) in bacterial (ANOVA: $F = 59.22$, $df = 2$, $P < 0.001$, Fig. 2A) but not in fungal communities (ANOVA: $F = 1.228$, $df = 2$, $P = 0.303$, Fig. 2B). Finally, evenness measured by the Pielou index decreased significantly for bacterial communities (Pielou: $F = 37.96$, $df = 2$, $P < 0.001$, Fig. 2A) but increased significantly in fungal communities (ANOVA: $F = 3.752$, $df = 2$, $P = 0.032$, Fig. 2B).

The beta-diversity, obtained using a PCoA projection and the Bray–Curtis dissimilarity index, differed significantly between soil microbial modalities according to the 3 obtained communities’ structures in both bacterial (SES = 234.6, $P = 0.001$) and fungal communities (SES = 167.9, $P = 0.001$) (Figs. 2C and D, respectively). Frequencies of bacterial and fungal genera for each microbial modality are shown in supplementary data S2 and S3.

Plants

Roots Mean contents of each analyzed root metabolite are shown in supplementary data S4. Total AA content differed significantly between treatments (ANOVA: $\chi^2 = 11.157$, $df = 2$, $P = 0.004$, Fig. 3A): “healthy” plants had a greater AA content than “35 dai” plants (lsmeans: *t* ratio = 3.327, $df = 64$, $P = 0.012$) while content for “1 dai” plants was intermediate. However, soil microbial diversity did not significantly affect this content (ANOVA: $\chi^2 = 5.668$, $df = 2$, $P = 0.177$), although the same trend was observed, the “high” modality tending to be higher than the “medium” one.

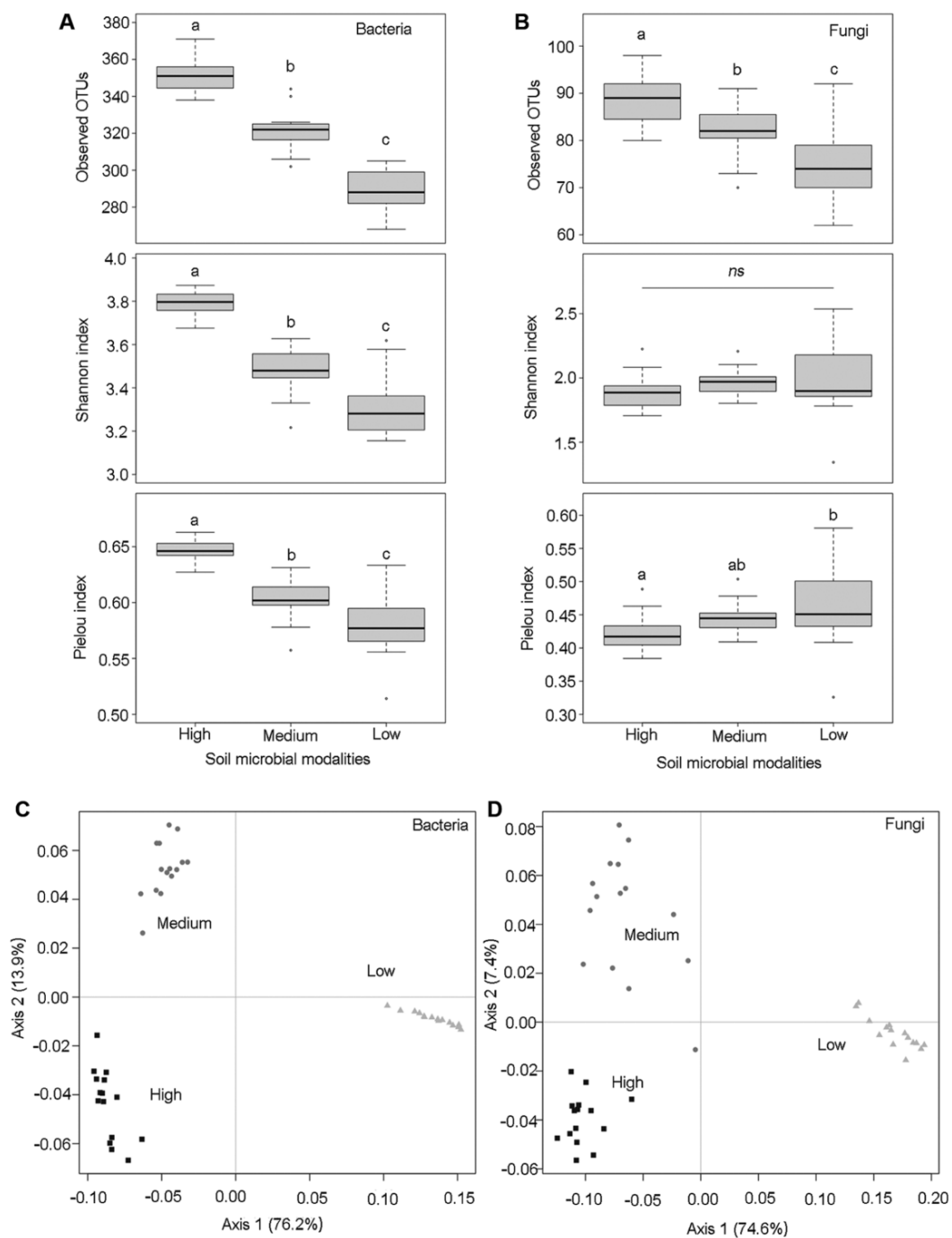
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Fig. 2 Indices (A and B) and communities' structures (C and D). Mean richness (number of observed OTUs), alpha-diversity (i.e., Shannon index) and evenness (i.e., Pielou index) for the 3 soil microbial modalities ("high," "medium," and "low") in bacterial (A) and fungal (B) communities. PCoA projection of the communities' structure (i.e., beta-diversity) using Bray–Curtis dissimilarity index for bacteria (C) and fungi (D), where "high," "medium," and "low" modalities are represented in dark, medium, and light gray, in addition to squares, circles, and triangles, respectively. Different letters indicate significant differences among communities. $N = 45$ samples, 15 per soil microbial modality.

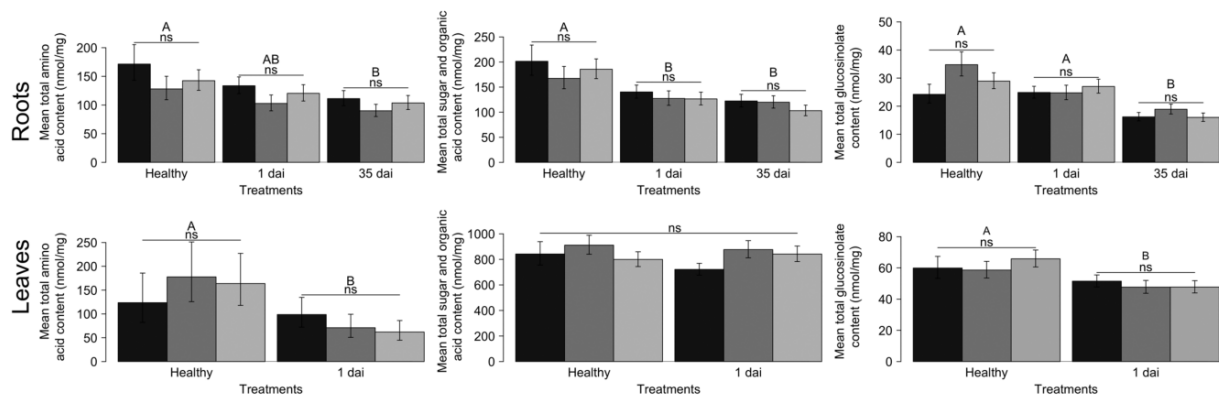


Fig. 3 Mean amino acid (A, D), sugar and organic acid (B, E), glucosinolate (C, F) contents (nmol/mg) in roots (A, B, C), and leaves (D, E, F) per soil microbial diversity (dark, medium, and light gray represent “high,” “medium,” “low” diversities, respectively) and per treatment (“healthy,” “1 dai,” and “35 dai”). Different letters indicate significant differences between treatments or soil microbial modality. (\pm SE, $N = 9$ plants per soil microbial modality for “healthy,” “1 dai,” and “35 dai” treatments of both roots and leaves).

Soil biological replicates were not significantly different (ANOVA: $\chi^2 = 1.987$, $df = 2$, $P = 0.555$). As AAs, total SOA content differed between treatments (ANOVA: $\chi^2 = 28.193$, $df = 2$, $P < 0.001$, Fig. 3B), “healthy” plants being higher than both infested treatments (lsmeans: t ratio = 3.862, $df = 63.21$, $P = 0.001$ and t ratio = 5.247, $df = 19.79$, $P < 0.001$ for “1 dai” and “35 dai,” respectively). Infested treatments did not differ significantly (lsmeans: t ratio = 1.645, $df = 17.53$, $P = 0.254$), nor did soil microbial modalities (ANOVA: $\chi^2 = 2.260$, $df = 2$, $P = 0.323$) and soil biological replicates (ANOVA: $\chi^2 = 0.981$, $df = 2$, $P = 0.612$). Total GSL content differed between treatments (ANOVA: $\chi^2 = 48.193$, $df = 2$, $P < 0.001$, Fig. 3C): “35 dai” plants were associated to lower content than “healthy” and “1 dai” plants (lsmeans: t ratio = 6.328, $df = 37.37$, $P = 0.001$ for “healthy”–“35 dai” and t ratio = 5.587, $df = 34.71$, $P = 0.001$ for “1 dai”–“35 dai”). The 2 latter were similar (lsmeans: t ratio = 1.507, $df = 63.13$, $P = 0.294$ for “healthy”–“1 dai”). No difference was observed between soil microbial modalities (ANOVA: $\chi^2 = 3.150$, $df = 2$, $P = 0.310$) but “healthy” plants seemed to display a higher GSL content at the “medium” than at the “high” modality. Again, soil biological replicates were similar (ANOVA: $\chi^2 = 2.898$, $df = 2$, $P = 0.555$).

Leaves Mean contents of each analyzed leaf metabolite are shown in supplementary data S5. Total AA content differed between treatments (ANOVA: $\chi^2 = 16.129$, $df = 1$, $P < 0.001$, Fig. 3D): “healthy” plants had higher contents than “1 dai” plants. However, there was no effect from soil microbial modalities (ANOVA: $\chi^2 = 1.013$, $df = 2$, $P = 0.643$). Total AA content differed marginally

between soil biological replicates (ANOVA: $\chi^2 = 8.201$, $df = 2$, $P = 0.051$). Curiously, none of the variables had an effect on total SOA content (ANOVA: treatments, $\chi^2 = 0.256$, $df = 1$, $P = 0.613$; soil microbial modalities, $\chi^2 = 4.667$, $df = 2$, $P = 0.291$; soil biological replicates, $\chi^2 = 4.406$, $df = 2$, $P = 0.165$, Fig. 3E). Total GSL content differed between treatments (ANOVA: $\chi^2 = 13.301$, $df = 1$, $P < 0.001$, Fig. 3F): “healthy” plants had higher contents than “1 dai” ones. However, no statistical difference were observed between soil microbial modalities (ANOVA: $\chi^2 = 0.883$, $df = 2$, $P = 0.643$) or soil biological replicates (ANOVA: $\chi^2 = 2.795$, $df = 2$, $P = 0.247$).

Insects

Oviposition Egg numbers ranged from 0 to 123, with a high heterogeneity between soil microbial modalities but also between plants from the same modality (mean 41.51 ± 5.99). No significant differences were found between soil microbial modalities (ANOVA: $\chi^2 = 0.231$, $df = 2$, $P = 0.890$, Fig. 4A), nor between soil biological replicates (ANOVA: $\chi^2 = 3.757$, $df = 2$, $P = 0.152$).

Development and fitness Adult emergence rates differed significantly between soil microbial modalities (ANOVA: $\chi^2 = 7.8216$, $df = 2$, $P = 0.020$, Fig. 4B) but not between soil biological replicates (ANOVA: $\chi^2 = 0.0083$, $df = 2$, $P = 0.995$). Emergence rate was higher at the “medium” than the “high” and “low” modalities (lsmeans: z ratio = -2.552 , $P = 0.032$ and z ratio = 2.273 , $P = 0.034$, comparison with “high” and “low,” respectively). For the 2 latter modalities, emergence rate was similar (lsmeans: z

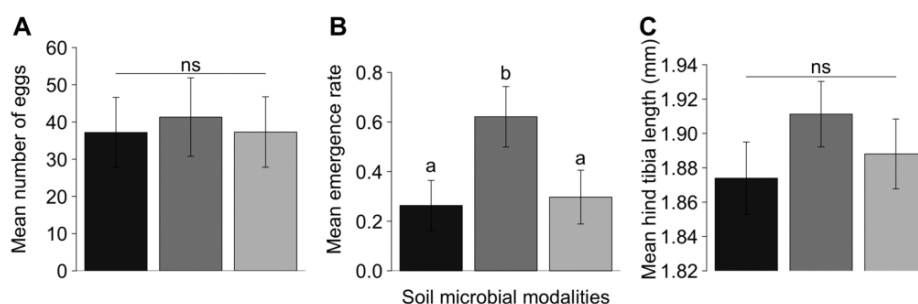


Fig. 4 Mean oviposition behavior (i.e., mean number of eggs, \pm SE, $N = 27$ plants per microbial modality A), emergence rate (\pm SE, $N = 27$ plants per microbial modality B) and length of the hind tibia (\pm SE, “high” modality $N = 72$ flies, “medium” $N = 120$, “low” $N = 81$ C) for the 3 levels of soil microbial modality (dark, medium and light gray represent “high,” “medium,” and “low” modalities, respectively). Different letters indicate significant differences among modalities.

ratio = -0.282 , $P = 0.777$). Fitness evaluated by hind tibia length did not differ significantly between soil microbial modalities (ANOVA: $\chi^2 = 4.045$, $df = 2$, $P = 0.132$, Fig. 4C) nor between soil biological replicates (ANOVA: $\chi^2 = 1.494$, $df = 2$, $P = 0.473$). However, we also noted that hind tibia length from the “medium” modality tended to be longer than in the “high” one.

Discussion

We showed that soil microbial diversity manipulation through serial dilutions creates a clear decreasing gradient of bacterial richness, as observed by Hol *et al.* (2010). In this previous work, microbial diversity loss had a linear and positive effect on the development of a phloem-sucking insect (i.e., aphids) and on plant production. Here we found that microbial diversity also influenced insect development but marginally plant biochemical profiles.

Delia radicum development quality was measured by counting adult offspring and evaluating their size. The CRF emergence rates are known to range from 45% to 78% depending on oilseed rape genotype (Hopkins *et al.*, 1999). The magnitude of the differences we observed is therefore significant: almost 30% more flies emerged from the “medium” microbial modality, which is comparable to differences previously observed among plant genotypes by Hopkins *et al.* (1999). Offspring size (estimated via hind tibia length) displayed the same trend, even though not significant, with a larger size at the “medium” microbial modality. Hind tibia length has been commonly used as a measure of adult size in many insects, and is correlated to emergence rate in CRF (Soler *et al.*, 2007). No effect was observed on oviposition. These observations on insect traits were associated with the tendency of total amino acid content in roots to be lower at the “medium”

microbial modality. Metabolite contents differed significantly between treatments, revealing infestation and time effects and a weak soil microbial effect in roots and an infestation effect in leaves. However, the effect of CRF infestation on root and leaf metabolites is debated. van Geem *et al.* (2015) observed an increase of GSL content in one *Brassica oleracea* population infested by CRF and no effect on total AAs and sugars in roots. Pierre *et al.* (2012) had the same result regarding AAs, but total root sugar content was lower in infested plants while no difference was observed on leaf metabolites.

Reported effects of AAs on insect development are also ambiguous. They could be a marker of plant nutritive quality: van Geem *et al.* (2015) found that concentrations of histidine, asparagine, glutamine, and serine in wild cabbage were positively correlated to CRF biomass. Alternatively, they could be indirectly involved in plant defenses by providing the energy needed to produce defensive compound (Bolton, 2009) or jasmonic acid (Howe & Jander, 2008). This latter compound is a signal transducer produced following herbivory, which increases concentrations of defense-related chemicals such as protease inhibitors, highly damageable for insects (Koiwa *et al.*, 1997). In our experiments, the plant defense boost provided by amino acids might outweigh their nutritional benefit to the insect. This could explain why insects develop better on plants grown on medium rather than high microbial soil diversity. Sugars are an easily metabolized source of energy and commonly seen as beneficial for insect development (Bernays & Chapman, 1994). For example, pupal weight is correlated with fructose, glucose, and total sugar content (Hopkins *et al.*, 1993, 1999). However, the similarity in total sugar content between microbial modalities we found at each stage of infestation suggests that sugars did not contribute significantly to the insect development differences we observed. GSLs

are secondary metabolites highly concentrated in *Brassica* species. While their deterrent and toxic properties toward many generalist insects have been emphasized (Fürstenberg-Hägg *et al.*, 2013), their effects on specialist insects are more ambiguous. Indeed, GSLs are oviposition and feeding stimulants for at least 25 species including Dipterans and the Brassicaceae specialist *D. radicum* (Hopkins *et al.*, 2009). The lack of oviposition preference in our study could be explained by the fact that we observed no change in contact cues such as leaf GSLs, AAs, and SOAs between soil microbial modalities. The lack of an alternative oviposition site might have also forced females to lay their eggs on whatever site was available. An experiment offering a choice between plants grown in each soil microbial modality could be more appropriate (see for e.g., Gouinguéné & Städler, 2006).

Even though rhizospheric microorganisms interact primarily with plants, their communities can also influence organisms further up the trophic chain, such as herbivorous insects. High microbial diversity in the rhizosphere has been associated with the activation of plant defense systems against pathogens (Berendsen *et al.*, 2012). Here, the “high” microbial modality (hence diversity) is associated with a lower insect emergence rate and with marginal changes in host–plant metabolic profiles. Through successive dilutions, we decreased the bacterial diversity where the pool of rare species is eroded as dilution progresses. This diversity gradient was not obtained for fungi because the decrease of fungi richness was accompanied by a rise of evenness, which made few genera of even abundance develop. Our results confirm the statement of Yan *et al.* (2015) that the dilution method is effective in obtaining a decreasing gradient in microbial OTUs richness, but that it allows no accurate predictions on the community structure and genera abundances. Soil bacterial richness has been shown to be of poor importance for mechanisms such as degradation of carbon sources or nitrogen cycling, since bacterial species are probably redundant in ecosystem functioning (see Roger *et al.*, 2016 for an overview of the bacterial diversity–ecosystem functioning literature including the dilution approach). However, we found a significantly better insect emergence at the “medium” diversity, which challenges this view of complete functional redundancy. We suggested that some of the rare species present in the “high” microbial modality are detrimental to insects, for example, by providing the plants with services not assumed by dominant genera. They could be involved in the activation and enhancement of plant defense against insect, as shown for some strains of bacteria and fungi (Pineda *et al.*, 2010). For instance, our study did not screen defensive compounds such as protein deaminases, phenols, or lectins (Felton

et al., 1999), which may be influenced by the plant microbiota and explain insect phenotypes. Insect development was also impaired in the “low” modality. Rare soil species being mostly absent here, the putative role of abundant microbial genera in insect life history traits should be considered. The genera *Pseudomonas*, *Hypocrea* (teleomorph of *Trichoderma*) and *Cordyceps* were significantly more abundant in the “low” modality. All 3 genera harbor entomopathogenic species and could adversely infect dipteran larvae such as *D. radicum* (Commare *et al.*, 2002; Zhang *et al.*, 2004; Razinger *et al.*, 2013). Other known entomopathogens are *Bacillus thuringiensis*, *Beauveria bassiana*, and *Metharhizium anisopliae*, which genera were detected in our samples, sometimes at very low frequencies (Damgaard *et al.*, 1997; Vänninen *et al.*, 1999; Razinger *et al.*, 2014). However, hypotheses about the potential role of these known pathogens must be made carefully here since the taxonomic resolution of the MiSeq Illumina sequencing technique is limited to the genus. It is therefore difficult to speculate about specific entomopathogenic species, which might not even be present. Moreover, the link between microbial diversity and entomopathogenic functions is poorly known and the pattern of dominant bacterial and fungal genera detected in our samples prevents from concluding on the role of particular microbial taxa on the plant and insect phenotypes observed in the “medium” modality. “Medium” diversity would be characterized by the absence of rare species inducing plant defenses and a lower abundance of dominant genera containing pathogenic species, explaining the better success of *D. radicum* development.

In conclusion, a global and multidisciplinary approach including microbial ecology, plant physiology, and entomology is a key tool in deciphering plant–microbe–insect interactions. Through the manipulation of their diversity, we have shown that diversity of soil microorganisms marginally influences plant metabolites and development of a belowground herbivore. Furthermore, studies on the dynamics of microbial structure and functions in the rhizosphere need to be performed, using different plant genotypes and during plant and insect development, in order to support these results and move from correlation to causalities.

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Disclosure

The authors declare no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

S1: Assessment of physicochemical characteristics of the soil used in our experiments in the native and experimental soils.

S2: Mean frequency (% \pm SE) per bacterial genera for each level of soil microbial modality: “high,” “medium,” and “low” represented by dark, medium, and light gray bars, respectively. Genera were retained if their frequency was above the threshold of 1%, taking “high” modality as a reference. Different letters indicate significant differences among communities with a significant threshold of 0.05. $N = 45$ samples, 15 per microbial modality.

S3: Mean frequency (% \pm SE) per fungal genera for each level of soil microbial modality: “high,” “medium,” and “low” represented by dark, medium, and light gray bars, respectively. Genera were retained if their frequency

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was above the threshold of 0.5%, taking “high” modality as a reference. Different letters indicate significant differences among communities with a significant threshold of 0.05. $N = 45$ samples, 15 per microbial modality.

S4: Mean content (nmol/mg \pm SE) of each analyzed metabolites in roots per treatment (“healthy,” “1 dai,” “35 dai”) and soil microbial modality (“high,” “medium,” “low”).

[†]GABA: gamma-aminobutyric acid, SMCSO: S-methyl cysteine sulfoxide.

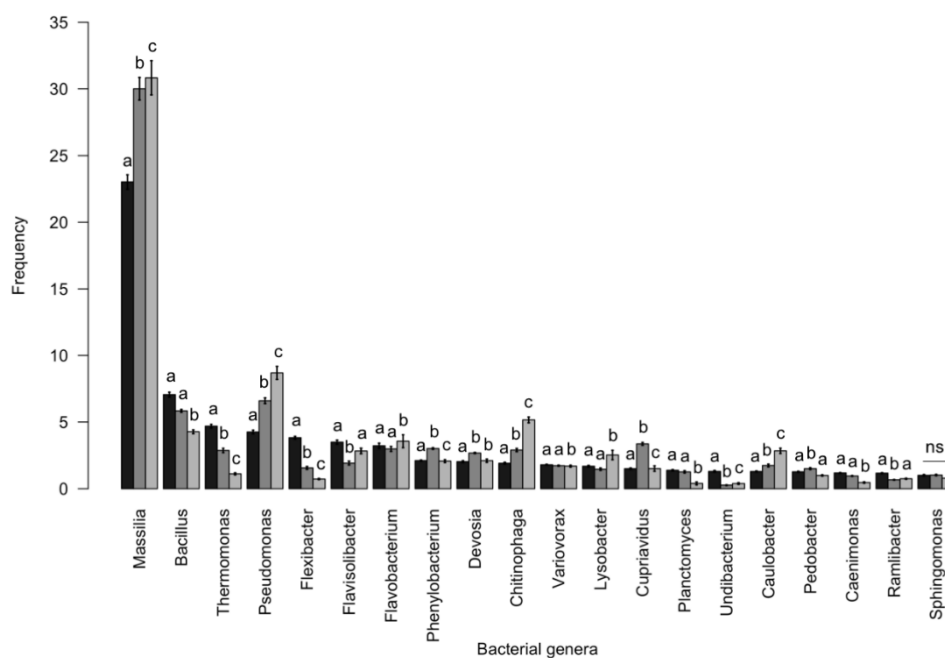
S5: Mean content (nmol/mg \pm SE) of each analyzed metabolites in leaves per treatment (“healthy,” “1 dai”) and soil microbial modality (“high,” “medium,” and “low”).

[†]GABA: gamma-aminobutyric acid, SMCSO: S-methyl cysteine sulfoxide.

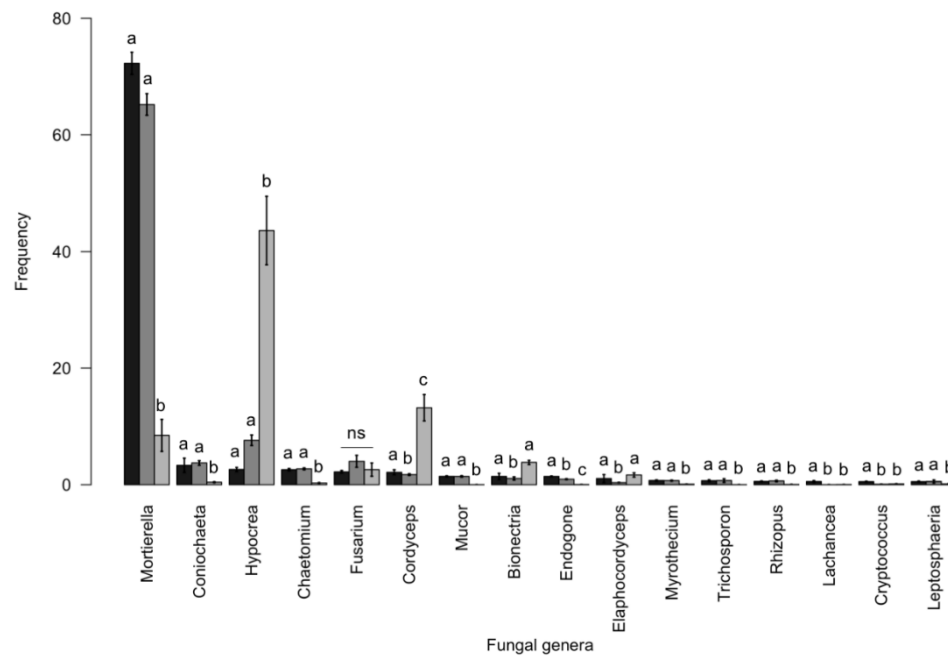
6. Supplemental materials

S1 Assessment of physicochemical characteristics of the soil used in our experiments in the native and experimental soils.

Physicochemical characteristics	Before incubation	After incubation
Sand	14.56%	36.36%
Silt	70.58%	52.28%
Clay	14.86%	11.37%
pH	6.11	6.16
Organic matter	19.78 g.kg ⁻¹	12.64 g.kg ⁻¹
Organic carbon	11.47 g.kg ⁻¹	7.31 g.kg ⁻¹
Mineral nitrogen	1.14 g.kg ⁻¹	0.77 g.kg ⁻¹



S2 Mean frequency (% \pm se) per bacterial genera for each level of soil microbial modality: 'high', 'medium' and 'low' represented by dark, medium and light grey bars respectively. Genera were retained if their frequency was above the threshold of 1%, taking 'high' modality as a reference. Different letters indicate significant differences among communities with a significant threshold of 0.05. N = 45 samples, 15 per microbial modality.



S3 Mean frequency (% \pm se) per fungal genera for each level of soil microbial modality: 'high', 'medium' and 'low' represented by dark, medium and light grey bars respectively. Genera were retained if their frequency was above the threshold of 0.5%, taking 'high' modality as a reference. Different letters indicate significant differences among communities with a significant threshold of 0.05. N = 45 samples, 15 per microbial modality.

S4 Mean content (nmol.mg⁻¹ ± se) of each analyzed metabolites in roots per treatment ('healthy', '1 dai', '35 dai') and soil microbial modality ('high', 'medium', 'low').

	Healthy			1 dai			35 dai		
	High	Medium	Low	High	Medium	Low	High	Medium	Low
Amino acids									
Alpha alanine	11.7 ± 2.01	7.55 ± 1.28	7.34 ± 1.06	11.7 ± 1.13	8.8 ± 0.74	10.56 ± 1.54	5.12 ± 0.46	4.13 ± 0.35	4.47 ± 0.43
Arginine	8.48 ± 2.21	3.12 ± 0.86	6.87 ± 0.88	8.19 ± 1.53	5.3 ± 1.8	6.8 ± 1.39	3.36 ± 0.79	3.13 ± 1.14	4.44 ± 0.72
Asparagine	5.44 ± 0.57	4.27 ± 0.61	4.68 ± 0.32	4.38 ± 0.73	3.22 ± 0.41	4.44 ± 0.63	4.34 ± 0.42	4.57 ± 0.86	4.33 ± 0.52
Aspartate	5.88 ± 0.57	4.61 ± 0.68	6.78 ± 0.73	4.76 ± 0.6	4.43 ± 0.91	4.92 ± 0.96	4.88 ± 0.72	5.64 ± 0.97	5.78 ± 0.87
GABA [†]	10.76 ± 1.41	8.02 ± 0.76	8.01 ± 1.13	10.29 ± 0.88	10.74 ± 1.14	10.66 ± 1.78	5.61 ± 0.33	5.51 ± 0.52	6.51 ± 0.76
Glutamate	9.76 ± 0.92	8.73 ± 1.03	9.85 ± 0.66	10.07 ± 1.27	8.43 ± 1.15	9.7 ± 1.28	11.58 ± 1.03	11.3 ± 1.38	11.51 ± 1.22
Glutamine	68.68 ± 11.43	51.89 ± 9.8	55.31 ± 5.19	48.53 ± 6.7	34.88 ± 6.33	42.39 ± 6.67	13.23 ± 1.34	12.5 ± 2.28	17.96 ± 2.54
Glycine	2.06 ± 0.37	1.75 ± 0.2	1.57 ± 0.19	1.78 ± 0.14	1.45 ± 0.18	1.7 ± 0.26	1.17 ± 0.22	0.91 ± 0.13	1.04 ± 0.18
Histidine	1.36 ± 0.21	0.98 ± 0.18	1.28 ± 0.11	1.12 ± 0.19	0.83 ± 0.15	0.99 ± 0.14	1.97 ± 0.28	1.58 ± 0.3	1.78 ± 0.24
Isoleucine	2.08 ± 0.34	1.85 ± 0.27	2.04 ± 0.17	1.8 ± 0.28	1.02 ± 0.13	1.15 ± 0.12	2.41 ± 0.23	2.01 ± 0.36	2.4 ± 0.32
Leucine	1.16 ± 0.26	1.14 ± 0.2	1.37 ± 0.17	1.51 ± 0.27	0.94 ± 0.15	1.1 ± 0.14	2.16 ± 0.29	1.8 ± 0.27	2.43 ± 0.34
Proline	8.93 ± 2.5	7.45 ± 3.09	12.76 ± 3.78	8.18 ± 1.09	3.88 ± 1.34	4.63 ± 0.98	29.99 ± 9.09	17.44 ± 8.39	19.96 ± 10.44
Serine	7.71 ± 1.05	6.09 ± 0.75	6.58 ± 0.47	6.77 ± 0.71	5.47 ± 0.48	6.35 ± 1	2.71 ± 0.31	3.63 ± 0.65	3.34 ± 0.33
SMCSO [†]	14.63 ± 0.72	13.34 ± 0.73	13.59 ± 0.87	14.09 ± 1.48	11.14 ± 1.04	12.55 ± 1.55	17.86 ± 1.54	16.88 ± 3.06	15.24 ± 1.55
Threonine	6.28 ± 0.55	4.87 ± 0.34	5.27 ± 0.3	5.24 ± 0.46	4.4 ± 0.43	5.1 ± 0.66	4.18 ± 0.35	4.37 ± 0.57	5.31 ± 0.6
Valine	4.86 ± 0.59	4.33 ± 0.51	4.71 ± 0.34	4.2 ± 0.48	2.77 ± 0.34	3.32 ± 0.4	6.32 ± 0.48	5.8 ± 0.75	6.44 ± 0.81
Sugars and organic acids									
Citrate	24.17 ± 5.36	12.43 ± 3.02	25.71 ± 3.98	19.73 ± 6.11	14.61 ± 3.62	17.25 ± 6.78	21.68 ± 3.89	23.41 ± 3.54	17.49 ± 5.12
Fructose	5.91 ± 0.79	6.53 ± 1.59	5.75 ± 0.62	6.91 ± 0.66	5.97 ± 0.76	6.35 ± 1.02	2.31 ± 0.7	2.23 ± 0.63	1.53 ± 0.2
Glucose	40.16 ± 3.6	35.73 ± 6.6	30.6 ± 2.49	32.11 ± 3.45	35.04 ± 3.73	31.5 ± 4.19	7.85 ± 1.78	7.24 ± 1.28	8.18 ± 0.7
Glycerate	2.19 ± 0.19	2.12 ± 0.38	1.76 ± 0.31	2.13 ± 0.38	1.66 ± 0.15	2 ± 0.33	1.69 ± 0.16	1.59 ± 0.17	1.19 ± 0.14
Malate	37.2 ± 5.18	31.85 ± 4.96	29.45 ± 4.42	26.11 ± 3.69	21.73 ± 2.2	22.22 ± 4.25	42.77 ± 3.86	44.38 ± 5.38	34.84 ± 3.15
Myo Inositol	5.29 ± 0.94	4.45 ± 1.24	6.44 ± 0.74	3.85 ± 0.4	4.12 ± 0.73	3.94 ± 0.69	2.89 ± 0.38	2.98 ± 0.51	2.09 ± 0.27
Saccharose	88.32 ± 14.76	82.33 ± 13.71	84.69 ± 8.02	55.41 ± 4.26	46.29 ± 6.89	52.26 ± 6.44	48.76 ± 5.13	45.24 ± 3.86	41.79 ± 3.52
Glucosinolates									
Glucobrassicinapin	15.19 ± 2.59	22.74 ± 2.37	18.78 ± 0.4	16.61 ± 1.01	17.46 ± 1.92	17.52 ± 0.74	1.66 ± 0.18	2.07 ± 0.33	1.72 ± 0.25
Glucobrassicin	2.77 ± 0.68	3.82 ± 0.6	2.58 ± 0.36	2.77 ± 0.35	2.26 ± 0.28	2.33 ± 0.11	9.58 ± 1.34	11.28 ± 1.45	10.02 ± 1.86
Glucanapin	1.84 ± 0.35	2.29 ± 0.22	2.11 ± 0.33	1.51 ± 0.17	1.28 ± 0.21	1.68 ± 0.21	0.64 ± 0.05	0.76 ± 0.1	0.61 ± 0.06
Progoitrin	4.68 ± 0.72	5.91 ± 0.32	5.47 ± 0.53	5.14 ± 0.46	4.62 ± 0.56	5.72 ± 0.47	4.88 ± 0.27	5.98 ± 0.59	4.76 ± 0.51

[†] GABA: gamma-aminobutyric acid, SMCSO: S-methyl cysteine sulfoxide.

S5 Mean content (nmol.mg⁻¹ ± se) of each analyzed metabolites in leaves per treatment ('healthy', '1 dai') and soil microbial modality ('high', 'medium', 'low').

		Healthy			1 dai		
		High	Medium	Low	High	Medium	Low
Amino acids	Alpha alanine	6.04 ± 3.84	120.75 ± 118.23	73.99 ± 71.5	2.23 ± 0.38	2.45 ± 0.7	1.92 ± 0.2
	Arginine	3.38 ± 2.11	2.68 ± 1.69	1.43 ± 0.28	1.61 ± 0.68	2.07 ± 0.86	1.3 ± 0.29
	Asparagine	4.94 ± 1.11	4.7 ± 1.46	4.01 ± 1.2	3.37 ± 0.96	3.31 ± 1.31	1.48 ± 0.56
	Aspartate	10.63 ± 2.01	11.31 ± 1.54	10.44 ± 1.11	9.8 ± 1.18	9.95 ± 2.43	7.61 ± 1.51
	GABA [†]	3.49 ± 1.9	1.12 ± 0.21	1.33 ± 0.16	1.18 ± 0.2	1.08 ± 0.25	0.81 ± 0.18
	Glutamate	17.39 ± 2.41	18.91 ± 2.42	18.53 ± 1.42	17.99 ± 1.69	17.35 ± 3.45	15.07 ± 1.41
	Glutamine	42.19 ± 19.13	33.32 ± 11.18	27.27 ± 8.44	22.44 ± 7.99	21.05 ± 9.41	11.3 ± 5.09
	Glycine	1.08 ± 0.5	0.91 ± 0.19	0.78 ± 0.1	0.76 ± 0.14	1.15 ± 0.45	0.67 ± 0.19
	Histidine	0.72 ± 0.31	0.56 ± 0.31	0.34 ± 0.14	0.48 ± 0.18	0.37 ± 0.15	0.18 ± 0.06
	Isoleucine	1.24 ± 0.43	1.18 ± 0.48	0.98 ± 0.24	1.03 ± 0.17	0.89 ± 0.23	0.72 ± 0.07
	Leucine	0.82 ± 0.16	1.06 ± 0.21	0.81 ± 0.15	1.07 ± 0.1	0.9 ± 0.16	0.87 ± 0.03
	Proline	24.76 ± 9.2	29.56 ± 10.81	29.69 ± 9.41	19.97 ± 5.33	14.55 ± 5.13	6.22 ± 1.75
	Serine	7.21 ± 0.78	6.96 ± 0.91	6.84 ± 0.85	7.18 ± 0.99	6.4 ± 1.54	5.16 ± 0.93
	SMCSO [†]	16.61 ± 2.79	15.74 ± 2.29	16.02 ± 1.65	14.13 ± 2.02	14.06 ± 3.19	9.02 ± 1.12
	Threonine	5.22 ± 0.88	4.1 ± 0.66	4.34 ± 0.6	4.19 ± 0.45	4.41 ± 1.11	3.23 ± 0.54
	Valine	3.31 ± 0.9	2.86 ± 0.82	2.58 ± 0.47	2.49 ± 0.33	2.17 ± 0.48	1.78 ± 0.15
Sugars and organic acids	Citrate	13.28 ± 4.25	9.04 ± 3.71	17.49 ± 4.81	18.84 ± 4.55	6.2 ± 1.98	15.17 ± 7.98
	Fructose	229.35 ± 23.83	250.39 ± 34.64	202.29 ± 13.66	174.72 ± 18.17	193.58 ± 26.48	188.97 ± 14.24
	Glucose	511.34 ± 74.14	593.17 ± 75.28	474.12 ± 41.2	431.49 ± 44.33	605.87 ± 42.1	553.01 ± 42.11
	Glycerate	1.28 ± 0.34	1.22 ± 0.11	1.72 ± 0.23	1.35 ± 0.1	1.31 ± 0.18	1.07 ± 0.2
	Malate	76.68 ± 18.29	59.92 ± 11.84	73.98 ± 10.63	83.9 ± 11.73	54.33 ± 5.5	67.27 ± 21.51
	Myo inositol	7.09 ± 1.33	6.02 ± 1.25	8.13 ± 1.11	7 ± 0.81	4.84 ± 0.64	5.18 ± 0.53
	Saccharose	20.56 ± 1.79	19.19 ± 1.07	25.68 ± 4.19	20.47 ± 1.08	24.68 ± 3.11	23.54 ± 1.18
Glucosinolates	Epiprogoitrin	8.95 ± 2.37	8.09 ± 0.91	8.97 ± 1.18	6.19 ± 0.69	6.04 ± 0.79	5.71 ± 0.85
	Glucobrassicin	1.79 ± 0.31	1.99 ± 0.48	1.31 ± 0.21	1.28 ± 0.27	1.19 ± 0.28	0.67 ± 0.21
	Gluconapin	36.22 ± 4.76	34.36 ± 2.4	42.3 ± 3.81	31.43 ± 1.37	29.14 ± 1.23	33.03 ± 3.84
	Gluconasturtiin	4.44 ± 0.63	4.24 ± 0.17	4.46 ± 0.31	4.32 ± 0.28	4.33 ± 0.31	4.51 ± 0.36
	Progoitrin	12.9 ± 2.72	11.02 ± 1.11	11.55 ± 1.3	8.01 ± 0.64	8.11 ± 0.94	7.04 ± 1.32

[†] GABA: gamma-aminobutyric acid, SMCSO: S-methyl cysteine sulfoxide.

Short note 1

Can soil microbial diversity influence a rhizophagous insect behavior through a change in plant visual and olfactory cues?

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Author contributions

MO, DC, CM and AMC conceived and designed research. LL and AYGE prepared the soil used in the experiment. MO carried out the oviposition experiment. MO, VC, LD, AYGE, LL, CP and CM participated to the eggs collection. CP collected the volatiles, which were analyzed by DC. MO and DC did the statistical analyses and interpreted data for the oviposition and olfactometry experiments respectively. MO wrote the manuscript that was commented and approved by all authors.

Abstract

Plant-insect interactions are known to largely affect the plant chemistry, where plants produce defenses to protect themselves from herbivorous insects but also emit various signals, attractive to insects, notably specialist ones, and oviposition stimulant. In the same time, soil microorganisms can play a major role to maintain plant health and to worsen insect development. Yet, no study unravels the effects of soil microorganisms on insect behavior. Our study aimed at assessing the effects of soil microbial diversity on *Delia radicum* behavior and evaluating whether it could be explained by visual and olfactory cues from oilseed rape (*Brassica napus*) measured at different plant development stages.

After manipulating soil microorganisms in order to obtain three levels of diversity which plants grew on, the fly oviposition was monitored in a choice experiment while leaf chlorophyll content (i.e. green color cue) and plant biomass were measured and the leaf VOC composition analyzed.

We showed that soil microbial diversity influenced the oviposition of *D. radicum* aboveground stage and female preference changed as the plant aged. These results could not be linked to the observed plant phenotype as leaf and root dried biomasses and VOC composition were not impacted by initial soil microbial diversity, while chlorophyll content slightly was.

In order to develop our understanding of the fly behavior, different plant cues should be assessed and especially contact ones like glucosinolates, well-known oviposition stimulants. Furthermore, disentangling chemicals associated to plants and to microorganisms could help to better comprehend the effects of each actor when studying a tripartite interaction.

Keywords: *Brassica napus*, *Delia radicum*, root herbivore, soil microorganisms, oviposition, VOCs, olfactometry, chlorophyll.

1. Introduction

Plant-insect interactions are known to largely affect the plant chemistry. In order to protect themselves against herbivores, plants can display different kinds of defenses (Fürstenberg-Hägg *et al.*, 2013; Aljbory and Chen, 2018). However, it has also been acknowledged that plants emit signals, attractive to insects, which sensitivity can depend on whether the insect is a generalist or a specialist. According to the work of Cáceres *et al.* (2016), a volatile organic compound (VOC) can repel a generalist on one hand, to the point of having an oviposition deterrence effect while the same VOC can attract a specialist on the other hand.

Over the years, researchers have found that microorganisms can play a major role to maintain plant health by improving their nutrition (Richardson *et al.*, 2009) or increasing their resistance to bioaggressors like pathogens or insects (Mendes *et al.*, 2011; Berendsen *et al.*, 2012; Shikano *et al.*, 2017). While several soil microbial species and even strains were highlighted to induce plant defenses that consequently affect herbivorous insects (Pineda *et al.*, 2010; Pangesti *et al.*, 2017), more researchers now focus on whole microbial communities. Indeed, several studies showed that manipulation of soil microbial communities induced changes in plant chemistry and on the development of insects (Hol *et al.*, 2010; Lachaise *et al.*, 2017; Ourry *et al.*, 2018) like phloem-sucking aphids or root chewing herbivore. These studies demonstrated that a relatively diverse microbial community tended to increase plant secondary metabolites, decrease primary metabolites and to negatively impact the insect fitness, such as size (Hol *et al.*, 2010) or emergence rate (Lachaise *et al.*, 2017). Consequently, these results question the female behavior, which is expected to choose the best host plant to guaranty a successful development to its offspring. From a field survey, Wennström *et al.* (2010) observed that larvae performed better with a higher survival rate on leaves from preferred host plants, which were lacking two chemical compounds detected in leaves of non-preferred host plants.

The cabbage root fly (*Delia radicum*) is a specialist of Brassicaceae crops and a root herbivore as a larva while its adult stage selects its host plant to lay its eggs based on the recognition of signals emitted by the plants, like contact and visual or olfactory cues (Gouinguéné and Städler, 2005; Gouinguéné *et al.*, 2006). For example, *D. radicum* flies react to chemical compounds like the glucosinolates and CIFs (Cabbage Identification Factors) present on leaf surface (Marazzi and Städler, 2004) and lay more eggs on plants that showed an increased accumulation of these compounds (Marazzi *et al.*, 2004; Marazzi and Städler, 2005; Gouinguéné and Städler, 2006a). Moreover, flies are sensitive to soil conditions, such as the

substrate composition or humidity (Košťál *et al.*, 2000), as well as shapes and colors (Degen *et al.*, 1999; Gouinguéné and Städler, 2005). Also, some VOCs released by plants are known to play a major role in the behavior of *Delia* species, which lay more eggs when detecting these compounds (Gouinguéné and Städler, 2006b; Kergunteuil *et al.*, 2012).

So far, no study unravels the effects of soil microorganisms on insect behavior and plant cues. In our study, we assessed the effects of soil microbial communities on *D. radicum* behavior and evaluated whether the fly behavior could be i) explained by visual and olfactory cues from oilseed rape plants (*Brassica napus*) and ii) stable over time with different development stages of the plant. Using a dilution to extinction method, we manipulated soil microbial diversity in order to obtain three levels of diversity on which the plants grew to reach two different development stages. The fly oviposition was monitored on these plants in a choice experiment while leaf chlorophyll content (i.e. green color cue) and plant biomass were measured and the leaf VOC composition analyzed.

2. Materials and Methods

2.1. Biological models

A batch of soil was collected in November 2014 from the layer –10 to –30 cm deep of a field in Brittany (La Gruche, Le Rheu, France, 48°08'44"N, 01°47'97" W) where wheat was cultivated for 20 years, and it was stored in containers at ambient temperature in the dark. After a year of storage in containers, we followed the protocol described by Lachaise *et al.* (2017) and Ourry *et al.* (2018) in order to obtain soils of different microbial diversity (high, medium and low).

Seeds of the same *Brassica napus* L. cultivar (subsp. *oleifera* cv. Tenor) were sown in the soil of each microbial diversity, resting on a layer of pozzolan. Plants used for the experiment 1 and 2 were respectively grown under natural late 2016 winter photoperiod (mean temp. 15.3°C, min/max 5.5/25.6°C) for four weeks and 2015 winter photoperiod (mean temp. 14.7 °C, min/max 5.8/22.1 °C) for 5 weeks in a greenhouse. Then they were moved to a climatic room (20±1°C day, 18±1°C night, L16:D8 and 80±10% relative humidity) until the experiments took place. Once a week, they were supplied in Hoagland solution by sub-irrigation (Hoagland and Arnon, 1950). The plants used in our experiments looked similar as they were all about 8-9 leaves old and were placed randomly in the greenhouse and climatic room.

The cabbage root flies (*Delia radicum*) used in our experiment came from a laboratory population, which originates from the field where pupae were collected in 2015 in Le Rheu (Brittany, France) and then reared in the conditions described by Lachaise *et al.* (2017) and Ourry *et al.* (2018). Fertilized females, aged of 9-12 days, were used.

2.2. Experimental designs

Experiment 1 To study fly oviposition, preliminary experiments (results not shown) were performed to select the optimal number of females (3 vs 5 vs 7 per plant) and the oviposition time (6 vs 24h). We selected the “5 females per plant and 6h” condition based on absence of non-oviposition and homogenous egg number laid on turnip roots (*Brassica rapa* L. subsp. *rapa*). Therefore, fifteen females were placed in a cage during 6 hours with three plants, one of each soil diversity level (figure 1A). The experiment was replicated fifteen times, with plants of each microbial diversity placed randomly in the cage (figure 1B), and performed on 4 and 6 week-old plants. Females were removed from the cage at the end of the 6h period and chlorophyll content was measured on the previously used plants at 15 spots covering two intermediate leaves. Then, the plant roots and soil were washed using the system described in figure 1C, in order to retrieve and count the eggs. Finally, roots and leaves were dried before assessing biomass.

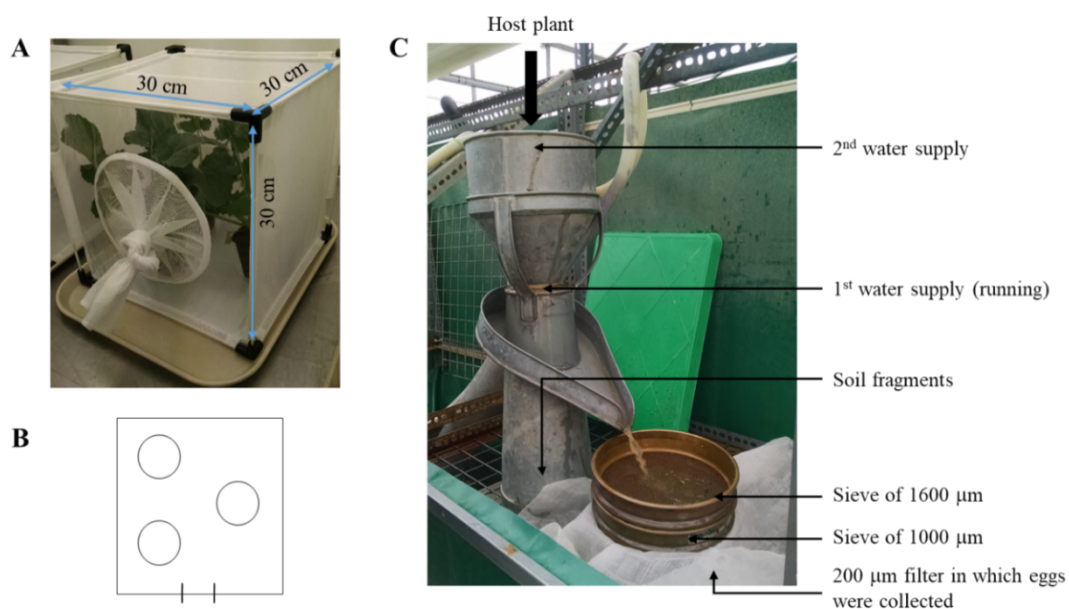


Figure 1 Materials for the oviposition experiment. (A) Photograph of a cage containing three plants grown on high, medium and low soil microbial diversities, and 15 *D. radicum* females. (B) View of the cage from above where plants, represented by circles, were randomly positioned inside the cage, with the cage entrance located at the bottom of the illustration. (C) Method to collect eggs laid on the plant crown and surrounding soil with accuracy. The host plant was washed with the second water supply. Heavy fragments (soil) went down into the container filled by the first water supply while light fragments (sand and fly eggs) floated and passed through the sieves.

Experiment 2 The VOCs were captured from another batch of 7 to 9 week-old healthy plants for each soil microbial diversity ($N = 9$ samples for high and medium, 7 samples for low diversity) using the design illustrated in figure 2. VOCs were condensed using a thermal desorber (TurboMatrix 350, PerkinElmer) and were analyzed by a gas chromatography (GC-2010 IMMO-009735, Shimadzu) coupled with mass spectrometry (GCMS-QP2010S, Shimadzu) and the GCMS Solution software (Version 4.3, Shimadzu). The GC column used had the following characteristics: SH-Rxi-5ms (Rxi: Restek's Exceptionally Inert); Length=30m; ID=0.25 mm; $df=0.25\ \mu\text{m}$ (Shimadzu). The 20 most expressed VOCs were identified by comparing their spectrum to the National Institute of Standards and Technology database.

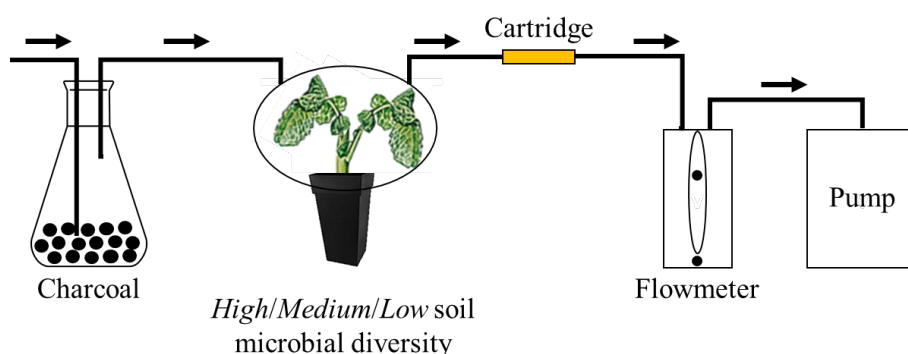


Figure 2 Olfactometry design to collect volatile organic compounds from oilseed rape plants. Arrows show the airflow that starts from the pump, through a flowmeter ($300\ \text{mL}\cdot\text{min}^{-1}$), charcoal (removes impurities from the air) and the plant that is emitting volatile organic compounds that are captured by the cartridge (TENAX, stocked at 4°C before and after using) during an hour. Another flowmeter closes the system in order to verify that the same amount of air is circulating from the beginning to the end of the system.

2.3. Statistical analyses

Analyses were performed using the R software (R Core Team, 2018) and a 5% threshold for statistical significance.

The number of eggs were converted into proportions of eggs laid on each soil microbial diversity. For both 4 or 6 week-old plants, female fly oviposition was analyzed with a Wald test that compared egg proportions explained by the initial soil microbial diversities.

Leaf and root dried biomasses as well as the chlorophyll content were analyzed separately for 4 and 6 week-old plants using a linear model and the following formula:
 $y \sim \text{Initial soil microbial diversity} + \text{Soil microbial diversity replicate} + \text{Plant replicates}$

The composition of the leaf VOCs was analyzed using a powered partial least squares discriminant analysis (PPLS-DA) after scaling the data and using the initial soil microbial diversity as the explanatory variable and also using a Principal Coordinate Analysis (PCoA, Hervé, 2016a, 2016b; Hervé *et al.*, 2018).

3. Results

Oviposition We observed that 31.3%, 30.2% and 38.5% of the eggs were laid on 4 week-old plants cultivated on high, medium and low soil microbial diversities respectively and the proportion of eggs was significantly lower on plants grown on high and medium microbial diversities and higher on the low microbial diversity. On 6 week-old plants, 30.8%, 38.8% and 30.4% of the eggs were found on the high, medium and low diversities respectively. This time, the proportion was still lower on plants grown on high microbial diversity as well as on the low diversity but higher on the medium microbial diversity (table 1, figure 3A).

Biomass Plant leaf and root dried biomasses were not influenced by the initial microbial diversity, no matter the plant development stage (table 1, figure 3B).

Chlorophyll However, soil microbial diversities increased lightly the chlorophyll content of plants grown on low microbial diversity, meaning the plants were slightly greener compared to the ones grown on medium diversity, but only at 4 weeks of development (table 1, figure 3C). For this trait, we observed an effect of the soil microbial replicate and the plant replicate, respectively for 4 and 6 week-old plants.

Table 1 Outputs of the statistical analyses performed on the oviposition and plant phenotypic traits. Significant results are identified in bold writing.

Development	y	Initial soil microbial diversity	Soil microbial diversity replicate	Plant replicate
4 week-old plants	Oviposition	$P_{\text{High-Medium}} = 0.536$ $P_{\text{High-Low}} < 0.001$ $P_{\text{Medium-Low}} < 0.001$	—	—
	Leaf biomass	$F=2.554$; $df=2$; $P=0.090$	$F=0.918$; $df=2$; $P=0.407$	$F=2.084$; $df=1$; $P=0.156$
	Root biomass	$F=1.680$; $df=2$; $P=0.199$	$F=0.586$; $df=2$; $P=0.561$	$F=1.438$; $df=1$; $P=0.237$
	Chlorophyll content	$F=7.071$; $df=2$; $P<0.001$	$F=7.695$; $df=2$; $P<0.001$	$F=3.788$; $df=1$; $P=0.052$
6 week-old plants	Oviposition	$P_{\text{High-Medium}} < 0.01$ $P_{\text{High-Low}} = 0.828$ $P_{\text{Medium-Low}} < 0.01$	—	—
	Leaf biomass	$F=1.214$; $df=2$; $P=0.307$	$F=0.953$; $df=2$; $P=0.394$	$F=1.927$; $df=1$; $P=0.173$
	Root biomass	$F=3.024$; $df=2$; $P=0.060$	$F=3.058$; $df=2$; $P=0.058$	$F=5.606$; $df=1$; $P=0.022$
	Chlorophyll content	$F=2.921$; $df=2$; $P=0.054$	$F=7.750$; $df=2$; $P<0.001$	$F=6.049$; $df=1$; $P=0.014$

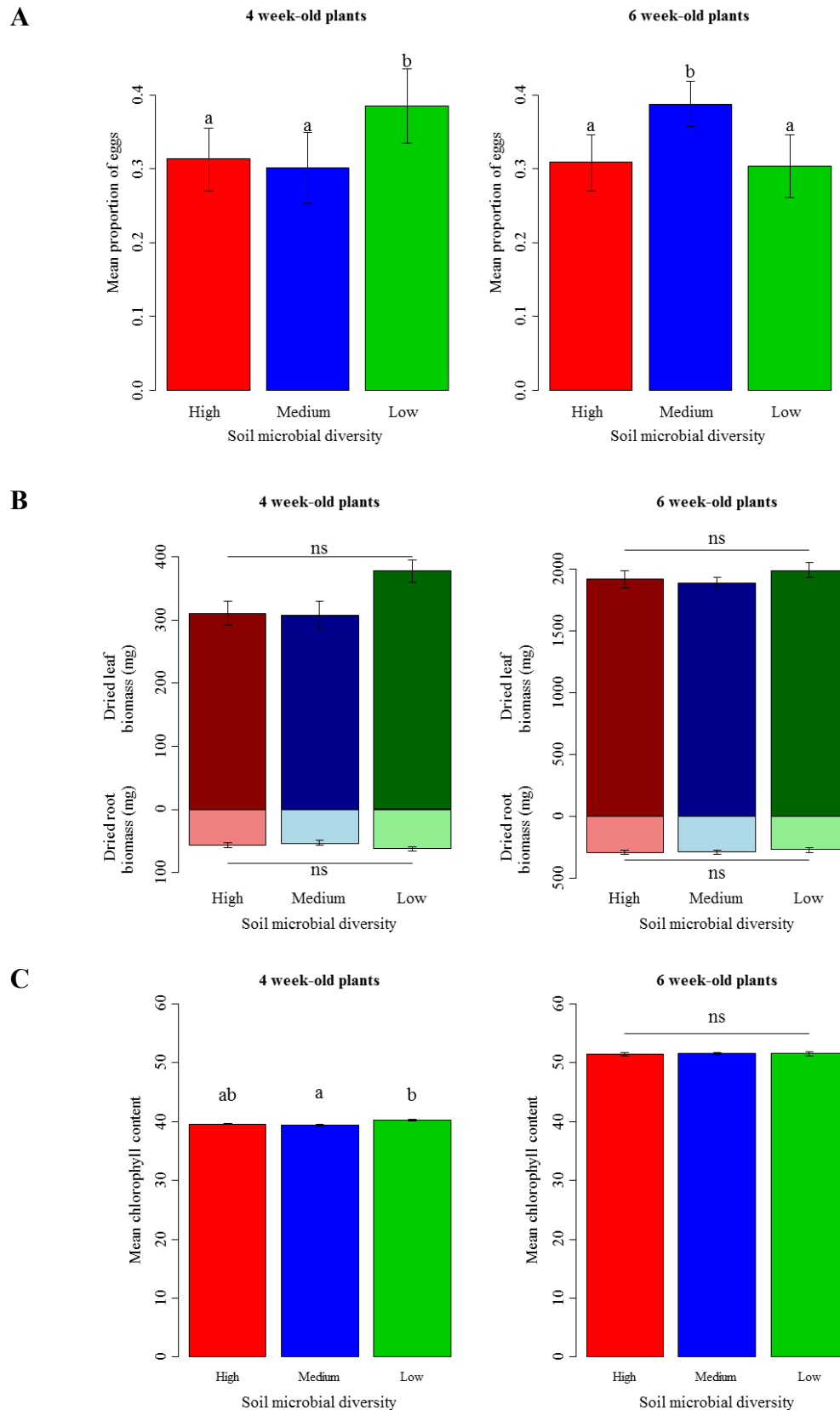


Figure 3 Fly oviposition behavior (A), leaf and root dried biomass (B) and chlorophyll content (C) per initial soil microbial diversity, assessed on 4 and 6 week-old plants. N = 15 plants per soil microbial diversity. The error bars correspond to standard errors. Significant differences are represented by lowercase letters.

Volatiles A total of 190 VOCs were detected in the leaves among all our samples with 8 to 20 compounds per sample. The PPLS-DA analysis indicated that leaf VOC composition was not significantly influenced by the initial soil microbial diversities (CER = 0.64, P = 0.551), even if three profiles seemed to be distinguished (figure 4A). However, a PCoA showed that the profile associated to each soil microbial diversity overlapped with each other (figure 4B).

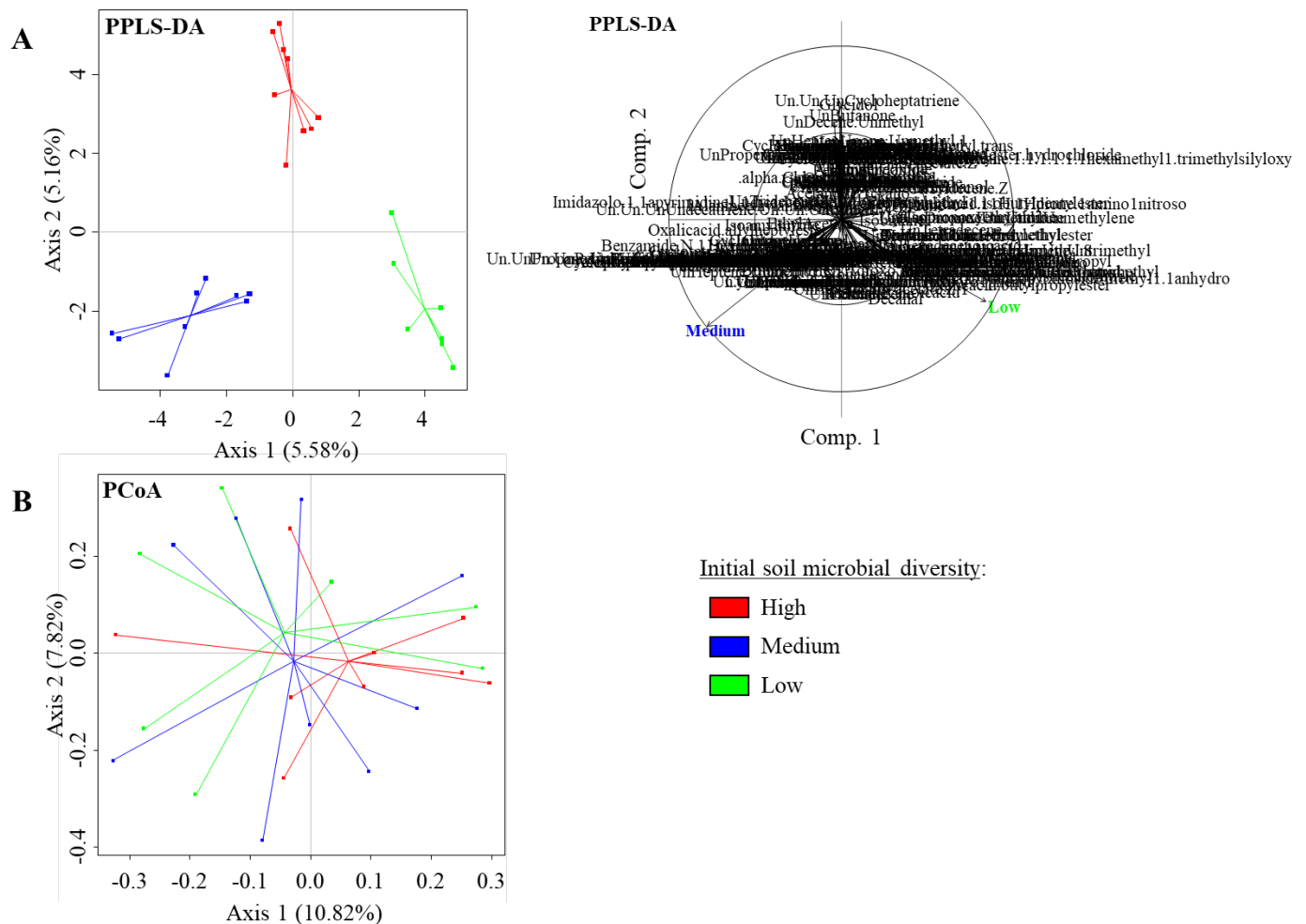


Figure 4 Multivariate analyses performed on leaf VOC composition of each soil microbial diversity. **(A)** Sample projection (plot on the left) and VOC correlation circle (plot on the right) obtained with PPLS-DA. **(B)** Sample projection obtained from a PCoA.

4. Discussion

Oviposition was influenced by the initial soil microbial diversity and changed over time as plant aged. After 4 weeks of plant cultivation, the female flies preferred to lay their eggs on plants grown on low diversity, which were also greener than the medium modality. Some studies demonstrated that *D. radicum* prefers visual cues such as oval shape and green colors (Gouinguené and Städler, 2005), which seems to be in agreement with our results. A large

number of chlorophyll measurement must be performed in order to obtain robust results and we ought to be careful when interpreting, as the differences between chlorophyll content were quite small. These results could not be linked to plant biomasses and VOC composition as they were not affected by microbial diversity. Similarly, at 6 weeks of plant cultivation, there were more eggs on plants cultivated on the medium diversity, with no relation to any of the measured plant traits. Therefore, the chlorophyll content is a trait insufficient to explain the fly oviposition in our study.

In the literature, several cues have been described as being attractive to the fly: specific substrate characteristics (Košťál *et al.*, 2000); volatiles (Gouinguéné and Städler, 2006b; Kergunteuil *et al.*, 2012); CIFs (Cabbage Identification Factors) or glucosinolates (GSLs) such as sinigrine, which are oviposition stimulant and considered as contact cues (Städler *et al.*, 2002; Gouinguéné and Städler, 2006a).

Regarding GSLs, they can be influenced by the initial soil microbial diversity and their total content increased with microbial diversity while nutrients like total amino acids and sugar decreased (Hol *et al.*, 2010). But these results were not found back by Lachaise *et al.* (2017) and Ourry *et al.* (2018), and GSLs were not measured in our study. The work of Fieldsend and Milford (1994) demonstrated that total GSL content of *B. napus* slowly increased until the end of pod formation but that GSLs were allocated in the different plant compartments, which content varied over time. These authors showed that leaf GSL content increased during the vegetative stage until green bud formation and then decreased but no measurement was performed on roots, which is the organ the cabbage root larva feeds on in our case. Another work focused on the evolution of GSLs in *Brassica juncea* cotyledons where the decrease of GSLs over time was associated with the enhancement of *Spodoptera eridania* larval weight and survival (Wallace and Eigenbrode, 2002). Therefore, we could suppose that a chemical remobilization occurs as the plant grows older, and more precisely a shift in the plant defenses, making the medium soil microbial diversity plant more attractive to female flies and potentially more palatable for the future offspring than plants grown on low microbial diversity.

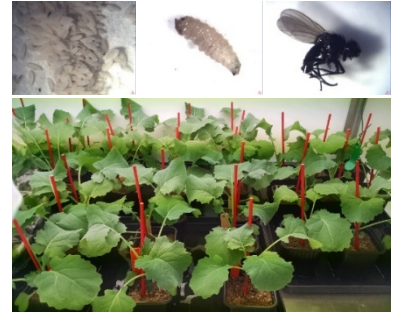
Moreover, plant VOCs can either act as oviposition deterrent or attractant depending on whether an insect is a generalist or specialist (Cáceres *et al.*, 2016). Yet, we did not manage to establish a relationship between the oviposition modulated by soil microbial diversity and VOC composition. In our study, we only captured leaf VOCs as the aboveground part of the plant was enclosed in a bag starting from the stem base, which did not allow the root VOCs to be taken into account. Also, VOCs of microbial origin should not be neglected as bacteria can enhance the plant defenses such as GSLs and be detrimental to insects (Aziz *et al.*, 2016) but

our experiment was not designed to disentangle plant VOCs from microbial ones, especially knowing that soil microbiota can be attracted by root VOCs (Schulz-Bohm *et al.*, 2018). Therefore, compounds from different plant compartments (from roots to leaves through cotyledons) and potentially from microorganisms could play a role in the fly behavior. To our knowledge, no study assesses the attractant or repellent properties of *B. napus* root and/or soil microbial VOCs toward herbivorous insects.

To conclude, our study showed that soil microbial diversity influenced the oviposition of *D. radicum* aboveground stage, which could not be linked to the observed plant phenotype. In order to develop our understanding of the fly behavior, different plant cues should be assessed and especially contact ones. As well, the different plant compartments should be considered when analyzing the plant chemistry and chemical analysis should be deepened by targeting relevant chemical compounds, known for their impact on the fly behavior. Further researches should be conducted to disentangle chemicals associated to plants and to microorganisms so that the effects of each actor could be better comprehend when studying a tripartite interaction.

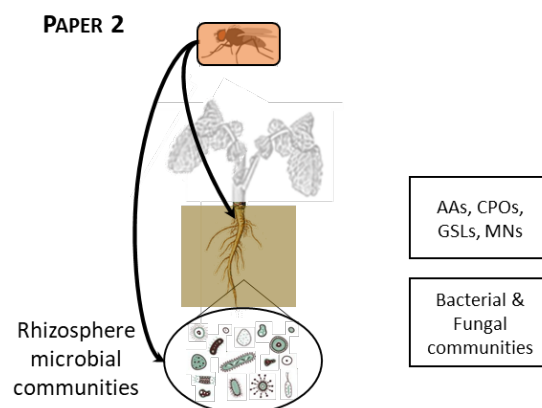
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CHAPTER III

ROOT HERBIVORY, A DRIVER OF PLANT CHEMISTRY AND MICROBIAL COMMUNITIES BELOWGROUND?



How does root herbivory by *D. radicum* modify plant root chemistry and microbial communities?

CHAPTER III: ROOT HERBIVORY, A DRIVER OF PLANT CHEMISTRY AND MICROBIAL COMMUNITIES BELOWGROUND?

Presentation of the chapter

Context

Disturbances are known to be important drivers of ecosystems and communities' dynamic on larger but also smaller scales such as microbial communities. Insect herbivory can also be seen as a disturbance. Plant-insect interactions have been largely studied especially concerning the defense mechanisms implemented by the plant against herbivorous insects and several studies have highlighted the implication of beneficial plant-microorganism interactions into shaping these defenses against insects. However, the reverse interaction is poorly studied. If it is known that insect herbivory modifies the plant physiology and chemistry, effects on microorganisms are not very documented.

Approach

In this study, our aims were i) to assess the effects of insect root herbivory on plant chemistry and microbial communities during the insect development using oilseed rape (*Brassica napus*) and the cabbage root fly (*Delia radicum*) and ii) to determine potential relationships between chemical and microbial modifications following herbivory.

As described in the previous chapter, we used the dilution to extinction method to obtain two soils of high and low microbial diversities (i.e. 10^0 and 10^{-6} dilutions respectively), where the plants grew on for six weeks before the beginning of the experiment. Initial soil microbial diversities were analyzed just before sowing with Illumina MiSeq sequencing and 16S and 18S primers, to assess bacterial and fungal communities respectively.

Root chemistry (amino acids, AAs; carbohydrates, polyols, organic acids, CPOs; glucosinolates, GSLs and chemical elements) and both root and rhizosphere bacterial and fungal communities were measured in healthy and *Delia*-infested plants, sampled during *D. radicum* development. The key development stages that we targeted were: hatching (1 day after infestation, dai), third instar larvae (14 dai) and end of adult emergence (42 dai), which respectively corresponded to the start, middle and end of the plant-insect interaction.

Main results

Our study showed that major changes induced by *D. radicum* to the plant chemistry intervened at the stage where the plant-insect interaction was the strongest: the third instar larval stage. Most metabolites and chemical elements decreased while two AAs (β -alanine, SMCSO), CPOs (trehalose, glycerate) and GSLs (glucobrassicin and neoglucobrassicin) out of seven increased in infested plants. Most of these influenced compounds recovered a level similar to the one of healthy plants at the end of the interaction and the initial soil microbial diversity had very little effect on the plant chemistry.

Diversity of bacterial and fungal communities were modulated by plant compartment (i.e. roots and rhizosphere), sampling time (i.e. 1, 14 and 42 dai), infestation status (i.e. healthy and *Delia*-infested) and initial soil microbial diversity (i.e. high and low). Bacterial communities were mainly composed of Bacteroidetes, Firmicutes and Proteobacteria phyla, and relative abundance of γ -Proteobacteria class significantly increased following herbivory, as Firmicutes but to a lesser extent. Similarly, we pointed out several bacterial genera, which abundance increased in infested plants, yet differently depending on the initial soil microbial diversity the plants grew on. These increases were notably observed in genera like *Bacillus*, *Paenibacillus*, *Pseudomonas* and *Stenotrophomonas*.

Regarding fungal communities, they were mainly composed of Ascomycota and Chytridiomycota, which abundances varied greatly but globally, fungi were less impacted than bacteria by the different factors we tested. This could be due to bacteria and fungi having different metabolisms, hence developing at different speed.

When looking for relationships between chemical and microbial data, we observed that the increased abundance of the above mentioned four bacterial genera were associated with the increase of trehalose, neoglucobrassicin and nitrogen contents during the peak of herbivory. Based on the literature, we suggested a potential scenario to explain these associations, hypothesizing in our study that plants produce defensive metabolites but also attract and recruit rhizosphere microorganisms through the roots using root chemical compounds in order to maintain their defenses against insect herbivory.

Limits

In our study, our analyses relied on three samples per condition. We wanted to perform four different extractions (DNA; amino acids; carbohydrates, polyols and organic acids; chemical elements) on the same plants, we realized that we did not have enough dried matter for all of these extractions. We initially had nine plants per condition, but we had to pool the

dried matter of three plants together, therefore ending with only 3 replicates per condition. For a stronger statistical power, it would have been better to either produce more plants or select the most relevant extractions.

Perspectives

It would be more relevant to differentiate plant chemical compounds from the ones produced by microorganisms to understand the mechanisms causing microbial community changes. Such data would allow reduction of potential biases in interpretations. Similarly, taking into account root exudates as modulators of microbial communities would improve our understanding of this tripartite interaction.

Establishing relationship between chemical and microbial data using a statistical analysis was interesting but rather incomplete and acquiring functional data, even with predictive tools, would have filled the gap and make us go further in our interpretations.

Publication status

Paper 2 was published as a scientific paper, entitled “Influence of Belowground Herbivory on the Dynamics of Root and Rhizosphere Microbial Communities” in the 2018 special issue of *Frontiers in Ecology and Evolution*: “As Above so Below? Below-ground Interactions in Ecological Processes”.



Influence of Belowground Herbivory on the Dynamics of Root and Rhizosphere Microbial Communities

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Recent studies are unraveling the impact of microorganisms from the roots and rhizosphere on interactions between plants and herbivorous insects and are gradually changing our perception of the microorganisms' capacity to affect plant defenses, but the reverse effect has seldom been investigated. Our study aimed at determining how plant herbivory influences the dynamics of root and rhizosphere microbial community assemblages and whether potential changes in root metabolites and chemical elements produced during herbivory can be related to microbial community diversity. We conducted our study on oilseed rape (*Brassica napus*) and its major belowground herbivore, the cabbage root fly (*Delia radicum*). We further assessed the influence of initial soil microbial diversity on these interactions. Different microbial diversities based on a common soil matrix were obtained through a removal-recolonization method. Root and rhizosphere sampling targeted different stages of the herbivore development corresponding to different perturbation intensities. Root bacterial communities were more affected by herbivory than some rhizosphere bacterial phyla and fungal communities, which seemed more resistant to this perturbation. Root herbivory enhanced the phylum of γ -Proteobacteria in the roots and rhizosphere, as well as the phylum of Firmicutes in the rhizosphere. Herbivory tended to decrease most root amino acids and sugars, and it increased trehalose, indolyl glucosinolates, and sulfur. Higher abundances of four bacterial genera (*Bacillus*, *Paenibacillus*, *Pseudomonas*, and *Stenotrophomonas*) were associated following herbivory to the increase of trehalose and some sulfur-containing compounds. Further research would help to identify the biological functions of the microbial genera impacted by plant infestation and their potential implications in plant defense.

Keywords: *Delia radicum*, *Brassica napus*, soil microbial diversity, rhizosphere and root microbial communities, herbivory, metabolites, chemical elements

INTRODUCTION

Interactions between plants and herbivorous insects are known to influence the evolution of plant defense mechanisms such as defensive and toxic metabolites or volatiles attracting natural enemies of the pest (Fürstenberg-Hägg et al., 2013; Nishida, 2014). Plants constitute an important crossroad in biological interactions since they are also involved in interactions with microorganisms that can improve plant growth, nutrient acquisition, and protection from different plant bioaggressors (Richardson et al., 2009; Schnitzer et al., 2011). Recent studies are unraveling the impact of microorganisms from the rhizosphere on plant-insect interactions and are gradually changing our perception of the microorganisms' capacity to affect plant defenses (Pineda et al., 2017). A single strain or a whole microbial community has been shown to increase plant defenses, which in turn can negatively impact insect fitness (Hol et al., 2010; Pangesti et al., 2015a,b; Lachaise et al., 2017). However, the reverse effect has seldom been investigated while herbivore attacks on plants could represent important perturbations for belowground microbial communities.

Perturbations are seen as major drivers of ecosystems stability (Milchunas et al., 1988). Since the 90's, the positive relationship between an ecosystem's stability and its diversity was addressed in different systems and particularly in grasslands facing different abiotic stresses (Ives and Carpenter, 2007). It seemed that the recovery of an ecosystem following a stress could be conditioned by the ecosystem's initial diversity prior to this stress. For example, an ecosystem with high plant diversity that suffers a drought will better recover than an ecosystem with a low plant diversity (Tilman and Downing, 1994; van Ruijven and Berendse, 2010). Effects of such stresses were investigated on resistance (the ability to sustain a perturbation or stress) and resilience (the capacity to come back to a stable state) of different soil processes such as nitrogen and carbon cycles, organic matter decomposition, or respiration (Griffiths and Philippot, 2013). Resistance and resilience of grassland communities toward biotic stresses such as herbivory has also been investigated (see review by Cingolani et al., 2005). However, these studies concerned either grazing mammals or insect herbivory in grasslands, with the latter having variable effects on mycorrhiza (Johnson and Rasmann, 2015). To our knowledge however, this question has never been addressed for microbial communities of agroecosystem plants confronted to insect herbivory.

Insect herbivory can modify plant physiology (Bolton, 2009) and alter metabolite concentrations in plant tissues (Griffiths et al., 1994; Hopkins et al., 1995; Ponzio et al., 2017) as well as nutrient uptake (Katayama et al., 2014; Aziz et al., 2016). It can also modify the release of organic matter by plant roots (rhizodeposition), a major driver of soil microbial communities (Singh et al., 2004; Paterson et al., 2007). Some studies showed

that root herbivory can influence plant carbon sources and rhizosphere chemistry, which in turn modify the abundance of bacteria and fungi, and community physiological profiles in the rhizosphere (Grayston et al., 2001; Dawson et al., 2004; Treonis et al., 2005). Katayama et al. (2014) showed that herbivory could also alter chemical element uptakes by the roots as well as nitrogen concentration of microbial origin. So far, when the effect of herbivory on plant-associated microorganisms was covered, the experiments either neglected to take into account the plant metabolites and chemical elements or did not integrate the notion of perturbation dynamics, or cultivable methods and fluorimetry were used to study microbial communities.

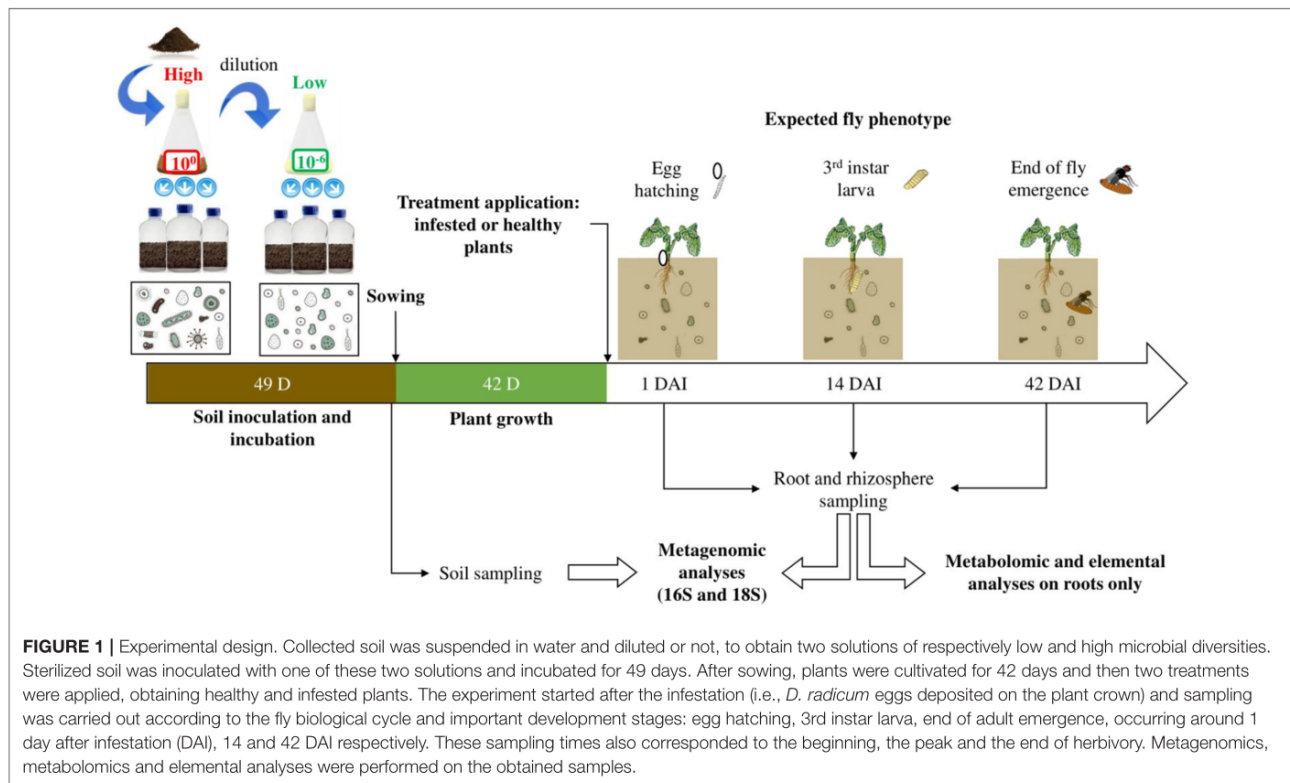
Our study aimed at determining the impact of insect herbivory on the plant-microorganism interactions. We hypothesized that (i) the dynamics of root and rhizosphere microbial communities would be influenced by belowground herbivory, (ii) these dynamics would depend on root metabolites and chemical elements induced by herbivory, (iii) the initial soil microbial diversity would influence plant chemistry and hence the dynamics of microbial communities. We conducted our experiment on oilseed rape ("OSR," *Brassica napus*) and its belowground pest, the cabbage root fly ("CRF," *Delia radicum*) (Ahuja et al., 2010). This fly is a specialist of brassicaceous plants. Females lay their eggs aboveground, at the base of plant stems and, upon hatching, which occurs a few days later, larvae feed by tunneling into the roots for 2–3 weeks, before pupating in the nearby soil and emerging. Herbivory of the CRF on brassicaceous plants is known to change the concentration of primary (e.g., sugars such as glucose and sucrose) and secondary metabolites (e.g., indolyl glucosinolates) in the roots (Hopkins et al., 1999; van Dam and Raaijmakers, 2006; Pierre et al., 2012; van Geem et al., 2015). However, nothing is known about how CRF herbivory and resulting biochemical changes in the roots influence the belowground microbial communities which interact with the plant.

MATERIALS AND METHODS

Soil Preparation and Inoculation

A batch of soil was collected in November 2014 from the layer –10 to –30 cm deep of a field in Brittany (La Gruche, Le Rheu, France, 48°08'44"N, 01°47'97" W) where wheat was cultivated for 20 years, and it was stored in containers at ambient temperature in the dark. After a year of storage in containers, the soil was homogenized, ground, sieved at 4 mm to remove the macrofauna and mixed with 1/3 silica. As described in Lachaise et al. (2017), this mixture was sterilized at 35 kGy and left 2 months to stabilize before inoculation while the remaining unsterilized soil with no silica was ground and sieved at 2 mm before being suspended in water. Following the detailed protocol of Lachaise et al. (2017), this suspension was then undiluted (10^0) or diluted at 10^6 before inoculating the sterilized soil, hence creating the two levels of soil microbial diversity used in our experiment: respectively "high" and "low" initial soil microbial diversities, also referred to as soil microbial modalities (Figure 1). This dilution-inoculation method was performed three times in order to obtain three soil biological replicates per soil microbial

Abbreviations: AA, Amino Acid; CPO, Carbohydrate, Polyol, and Organic Acid; CRF, Cabbage Root Fly; DAI, Days After Infestation; dbRDA, distance-based Redundancy Discriminant Analysis; DIABLO, Data Integration Analysis for Biomarker discovery using Latent cOmponents; FDR, False Discovery Rate; GSL, Glucosinolate; OSR, Oilseed Rape; RDA, Redundancy Analysis; SMCSO, S-Methyl Cysteine Sulfoxide.



modality. After inoculation, the soil was incubated in the dark for seven weeks at 18°C and 50% humidity. During this period, the bags containing the soil were opened weekly under sterile conditions using a laminar flow cabinet to homogenize the soil and facilitate microbial respiration and recolonization. This allowed the soil to reach optimal bacterial and fungal densities and similar abundances of Colony Forming Units between the two soil microbial modalities at the end of the recolonization period. A part of this soil was collected before sowing to evaluate initial soil microbial diversities ($N = 18$, 9 samples per soil microbial modality).

Insect Rearing

The population of cabbage root fly ("CRE", *Delia radicum*) used in our experiment was collected in the field in 2015 (Le Rheu, Bretagne, France). In the laboratory, flies were fed on sugar, milk powder and yeast (ratio 1:1:1) and they were reared on rutabaga roots (*Brassica napus* subsp. *rapifera*) in a climatic room (16:8 LD, $21 \pm 2^\circ\text{C}$; $60\% \pm 10\%$ RH) as described in Neveu Bernard-Griffiths (1998). Adult flies were left to oviposit on rutabaga roots for 24h and black-headed eggs (i.e., ready to hatch) were collected 3 days later for our experiment.

Plant Growth, Infestation, and Sampling

Seeds of *Brassica napus* L. (subsp. *oleifera* cv. Tenor) were sown in individual pots using a layer of pozzolan at the bottom and the soil previously obtained (characterized either with a "high" or "low" initial soil microbial diversity). Plants

were watered twice a week by sub-irrigation with a nutritive solution based on Hoagland and Arnon (1950) during the whole experiment. This solution was obtained by blending three separate solutions: a macronutrient solution (3 mM of KNO_3 , 0.5 mM of KH_2PO_4 , 1 mM of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2.5 mM of $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$), a micronutrient solution (10 μM of $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 1 μM of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 μM of CuSO_4 , 30 μM of H_3BO_3 , 1 μM of $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.5 μM of $\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$), and a Fe EDTA solution (27 μM of Fe EDTA Na). Plants were cultivated during 6 weeks in a greenhouse under natural late 2016 winter photoperiod (mean temp. 15.3°C , min/max $5.5/25.6^\circ\text{C}$). Two treatments were applied on the plants: half of the plants were infested by depositing eight black-headed eggs on the crown per plant, the other half remained untreated. These two batches of plants were then referred to as "infested plants" and "healthy plants" respectively. All plants were then moved to a climatic chamber (photoperiod 16:8 LD and thermoperiod 20:18°C LD) for the rest of the experiment. Afterwards, healthy and infested plants were sampled at 1, 14, and 42 days after infestation ("DAI"), which respectively represented (i) the hatching stage which corresponds to the initiation of herbivory, (ii) the third larval instar which corresponds to the peak of herbivory, and (iii) the end of the fly emergence which corresponds to the end of herbivory (Figure 1). A total of 108 plants were harvested, with 9 plants per condition (2 soil microbial modalities, 2 treatments being healthy and infested, and 3 sampling times). Roots and rhizosphere were sampled as follows: the root fraction was collected, corresponding to the area from the crown to the

tip roots, and was washed twice in 20 mL of sterile permuted water before being transferred to a clean Falcon tube; the whole root bath (i.e., 40 mL in a Falcon tube) corresponded to the rhizosphere fraction; both tubes were immersed in liquid nitrogen and stored at -80°C before being freeze-dried; only roots were ground using glass beads. In order to have sufficient material for molecular, metabolomic and elemental analyses, roots from three different plants of the same treatment, cultivated on the same soil microbial diversity and biological replicates, were pooled to make one sample, hence obtaining a total of 36 root samples ($N = 3$ samples per condition). The rhizosphere fraction ($N = 36$ rhizosphere samples) was treated similarly.

Molecular and Bioinformatic Analysis

Initial bulk soil (i.e., before sowing), as well as root and rhizosphere samples collected during the experiment, were analyzed.

According to the protocol developed by the GenoSol platform (Dijon, France) and described in Plassart et al. (2012), soil DNA was extracted from 2 g of wet bulk soil (i.e., initial soil before sowing) or 1 g of freeze-dried rhizosphere respectively in 8 or 5 mL of lysis buffer containing 100 mM of Tris-HCl (pH 8), 100 mM of EDTA (pH 8), 100 mM of NaCl, 2% SDS, and ultrapure water. The following modifications were performed: tubes were vortexed at mid and at the end of incubation (i.e., bath at 70°C), then centrifuged at 3,500 rpm at 15°C for 10 min; during deproteinization, samples were centrifuged at 14,000 g at 4°C during 10 min; during DNA precipitation, tubes were placed at -20°C for 30 min. To obtain DNA pellets, tubes were centrifuged at 13,000 rpm at 4°C for 30 min and supernatant was discarded. DNA pellets were washed as follows: 400 μL of 70% ice-cold ethanol were added to samples, which were centrifuged at 13,000 rpm at 4°C for 5 min and supernatants were removed. Remaining traces of ethanol were removed by heating open tubes at 60°C for at least 15 min or more if needed. Pellets of DNA were resuspended with 100 μL of ultrapure water and the duplicated samples were finally pooled. Bulk soil and rhizosphere samples were purified twice. The first purification required Microbiospin (Biorad, Hercules, California, USA) columns of PVPP (PolyVinyl PolyPyrrolidone, Sigma-Aldrich). To prepare the columns, their tips were removed and columns were placed in 2 mL Eppendorf tubes. Then, columns were washed with 400 μL of ultrapure water and centrifuged at 1,000 g and at 10°C during 2 min. After emptying the tubes, the previous step was carried out a second time, before centrifuging empty tubes at 1,000 g at 10°C for 4 min. Following Plassart et al. (2012), 100 μL of DNA were injected into the columns, previously transferred to a clean tube, however our samples were incubated on ice for 5 min, before a 4 min centrifugation at 1,000 g at 4°C . The obtained DNA ($\sim 95 \mu\text{L}$) was used for the second purification, performed using the GeneClean[®] Turbo kit (MP Biomedicals) with the following modifications: samples were centrifuged at 10,000 g for 10 s; after adding the GTE (GeneClean Turbo Elution Solution), samples were incubated on ice for 5 min and centrifuged at 10,000 g for 1 min; the GTE, incubation and centrifugation steps were repeated a second time, to finally obtain $\sim 60 \mu\text{L}$ of clean DNA.

Root DNA was extracted using the NucleoSpin[®] Plant II kit and protocol (Macherey-Nagel, Düren, Germany) with the following modifications: 30 g of freeze-dried root powder were used; (i) cell lysis was done with buffer PL1; (ii) incubation after adding RNase A lasted 30 min; (iii) the crude lysate was centrifuged before its filtration.

DNA quantification was performed using a Quantus fluorometer (Promega, Madison, Wisconsin, USA) and the Quantifluor kit (dsDNA: E2670).

PCR amplification and sequencing were performed at the GenoScreen platform (Lille, France) using the Illumina Miseq platform to a 2×250 bases paired-end version. We used PCR primer pairs 799F (5'-AACMGGATTAGATACCKG-3') and 1223R (5'-CCATTGTAGTACGTGTGA-3'), and NS22B (5'-AATTAAGCAGACAAATCACT-3') and SSU0817 (5'-TTAGCATGGAATAATRRATAGGA-3') (Lê Van et al., 2017) to amplify 16S and 18S rDNA genes, respectively.

To manage mismatch between reads 1 and 2 in the overlap region, we used bases trimming at Q30 with PRINSeq, trimming of specific primers with Cutadapt, assembly with FLASH starting with trimmed reads 1 and 2, a minimum of 30 bases overlapping was required with a 97% homology between reads. Regarding 18S rDNA, only read 1 was analyzed because i) amplicon size did not allow a sufficient overlapping area between read 1 and read 2 and ii) read 1 was of better quality. The GNS-PIPE bioinformatic pipeline was used for the bioinformatic analyses of 16S rDNA and 18S rDNA (Terrat et al., 2012). Raw data sets were deposited on the European Nucleotide Archive database system under the project number (PRJEB25217). Bulk soil samples accession numbers range from ERS2281263 to ERS2281298, those of root and rhizosphere samples range from ERS2255945 to ERS2256016 for 16S rDNA and from ERS2256770 to ERS2256841 for 18S rDNA.

Metabolites and Chemical Elements Analysis

Primary Metabolites: Amino Acids, Carbohydrates, Polyols, and Organic Acids

Quantification of free amino acids (AAs), non-structural carbohydrates, polyols, and organic acids (CPOs) was based on the method described by Gravot et al. (2010) and performed using 9 to 12 mg of freeze-dried root powder, with the same methanol-chloroform-water-based extraction. Minor adjustments were made to this protocol: after adding 100% chloroform, tubes were rapidly vortexed and then agitated for 10 min at room temperature; after adding water, samples were vortexed for 20 s and centrifuged for 5 min at 12,000 g and 15°C .

For AA derivatization and profiling, 50 μL of methanol-water extract were vacuum-dried. The dry residue was resuspended in 50 μL of ultrapure water and the tubes were rapidly vortexed, put in an ultrasonic bath for 5 min and centrifuged for 5 s at room temperature. Derivatization of AAs was performed using the AccQTag Ultra Derivatization kit (Waters) with the following modified volumes: 5 μL of the resuspended sample, 35 μL of AccQTag Ultra Borate Buffer and 10 μL of AccQTag Reagent were placed in a new tube, which was vortexed and placed

in a water bath for 10 min at 55°C. The whole volume was transferred in a vial and derivatized AAs were analyzed using liquid chromatography (Acquity UPLC-DAD system, Waters, Milford, MA, USA) according to Jubault et al. (2008). However, the column used for analyses was heated at 53°C and AAs were detected at 265 nm using a photodiode array detector. Identification of AAs was realized by comparison with a standard solution and quantification was made thanks to the internal standard BABA (3-aminobutyric acid).

Analysis of CPOs was performed by gas chromatography coupled to a flame ionization detector (GC-FID, Thermo Fisher Scientific, Waltham, MA, USA) and based on the protocol described by Lugan et al. (2009), which however required several modifications. The online derivatization for CPOs was performed with a Trace 1300 GC-FID (Thermo Scientific) equipped with a Tri Plus RSH (Thermo Scientific). Fifty microliters of the methanol-water extract were sampled in injection vials and vacuum-dried. This online derivatization was performed as follows: the dried extract was resuspended in 50 µL of pyridine containing 20 mg.mL⁻¹ of methoxyaminehydrochloride, under orbital shaking at 40°C for 90 min. Fifty microliters of MSTFA (N-methyltrimethylsilyltrifluoroacetamide) were added before incubation at 40°C for 30 min. One microliter of the mixture was injected into the GC-FID with a split/splitless injector (split mode set to 1:20) at 260°C, on a TG-5MS column (30 m × 0.32 × 0.25 mm, Agilent Technologies) connected to a flame ionization detector at 300°C. The temperature gradient of the GC oven was: 4 min at 100°C followed by an increase of 10°C.min⁻¹ up to 198°C and maintained at this temperature for 2 min; an increase of 1°C.min⁻¹ up to 202°C; then an increase of 15°C.min⁻¹ ramp up to 268°C and held for 3 min followed by an increase of 1°C.min⁻¹ up to 272°C and raised to 210°C at 10°C.min⁻¹ maintained for 7 min. Identification of CPOs was realized by comparison with a standard solution, while quantification was achieved with adonitol as the internal standard.

Secondary Metabolites: Glucosinolates

Extraction and analysis of glucosinolates (GSLs) were performed based on the protocol from Hervé et al. (2014) with the following modifications: GSLs were extracted from 12 to 15 mg of freeze-dried root powder and tubes were centrifuged for sedimentation at 12,000 g and 15°C for 5 min. Analysis of GSLs was performed using liquid chromatography coupled with mass spectrometry (Acquity UPLC-TQD, Waters) with electrospray ionization in a negative mode. Chromatographic conditions, A and B-elutents and the gradient used are described in Hervé et al. (2014). Quantification of GSLs was realized using an external calibration with a standard solution containing glucoerucin, gluconasturtiin, and glucobrassicin in the range of 4 to 80 µmol.L⁻¹. These compounds were respectively used to quantify aliphatic, aromatic and indolyl GSLs.

Chemical Elements

Analysis of root chemical elements composition was performed according to Maillard et al. (2015). About 4 mg of freeze-dried root powder were used to measure total N and S

contents, using a continuous flow isotope mass spectrometer (Nu Instrument, Wrexham, United Kingdom) linked to a C/N/S analyzer (EA3000, Euro Vector, Milan, Italy). For other elements such as K, Ca, P, Mg, Fe, Mn, Zn, Cu, Mo, Ni, and B, roots samples were submitted to microwave acid sample digestion (Multiwave ECO, Anton Paar, les Ulis, France) using 800 µL of concentrated HNO₃, 200 µL of H₂O₂ and 1 mL of Milli-Q water for 40 mg of root powder. All samples were previously spiked with two internal-standard solutions of Gallium and Rhodium, respectively, for a final concentration of 10 and 2 µg.L⁻¹. Mineralized samples were then diluted to 50 mL with Milli-Q water to obtain solutions containing 2.0% (v/v) of nitric acid, then filtered at 0.45 µm using a teflon filtration system (Filtermate, Courtage Analyses Services, Mont-Saint-Aignan, France). Samples were then analyzed by High Resolution Inductively Coupled Plasma Mass Spectrometry (HR ICP-MS, Thermo Scientific, Element 2TM) and quantification of each element was performed using external standard calibration curves. Additional information about mineralization and the utilization of HR ICP-MS can be found in the annexes of Maillard (2016).

Statistical Analyses

Analyses were performed using the R software (R Core Team, 2016) and a 5% threshold for statistical significance.

Microbial Communities

When analyses were performed on microbial data, some samples had to be removed from the dataset for the following reasons: one root sample was lost before sequencing (from the low diversity-healthy plants-14 DAI condition), one rhizosphere bacterial sample (from the high diversity-healthy plants-1 DAI condition) showed an abnormal smaller total read count after normalization while one root fungal sample (from the high diversity-healthy plants-1 DAI condition) had abnormal phyla abundances compared to other samples.

Bacterial and fungal richnesses and diversities, represented by the number of observed Operational Taxonomic Units (OTUs) and the Shannon index (obtained with the diversity function in the “vegan” package) respectively, were obtained from non-rarefied OTU data and analyzed in roots and rhizosphere separately using a linear model. Models took into account the following factors: sampling time (1, 14, and 42 DAI), plant treatment (healthy vs. infested plants), soil microbial diversity (high vs. low diversity), and soil replicate, as well as paired interactions between the three first factors. Linear models were adjusted depending on the significance of interactions: interactions were all removed if none was significant while they were kept if at least one was significant or close, which was respectively the case of analyses on the rhizosphere and root compartments. A type II analysis of variance table was performed on the models, followed by comparisons based on least-squares means when possible.

In order to analyze the bacterial and fungal community structure, distance-based redundancy discriminant analysis (dbRDA, dbRDA function in the “vegan” package) was performed on a Bray-Curtis dissimilarity matrix, obtained from OTU data,

which were filtered using a 1‰ threshold and log2-transformed. A type II permutation test for constrained multivariate analyses was performed on the dbRDA using the “RVAideMemoire” package (Hervé, 2016a,b) to evaluate the contribution of each factor (i.e., compartment, time, treatment, soil microbial diversity, and soil biological replicates) to microbial community structure. Quantified contributions expressed as *r*-squared, were obtained from the varpart function (“vegan” package).

Phyla analyses were performed according to the scripts provided by Bulgarelli et al. (2015) but *P*-values were corrected using the False Discovery Rate (FDR) method. Plotting was done with the “ggplot2” package.

Genewise negative binomial generalized linear models were conducted using the “edgeR” package (Anders et al., 2013) on filtered data to recover genera differences between plant treatments within sampling time. The obtained *P*-values were corrected with the FDR method. Using “ggplot2” package, genus count differences between infested and healthy (Xi – Xh) were plotted when the treatment was significant on one hand and when these differences exceeded 250 or –250 counts.

Metabolites and Chemical Elements

For each sampling time, two different analyses were performed on metabolites and chemical elements, taking into account the plant treatment (healthy vs. infested plants), the soil microbial diversity (high vs. low diversity), and the interaction between both factors, as well as soil biological replicates. Both analyses required the data to be transformed using the fourth root. First, a redundancy analysis (RDA), associated to a permutation significance test based on cross-validation, was performed to determine the influence of the previously mentioned factors on the root metabolomic and elemental profiles. Second, linear models were used to assess precisely which metabolites and elements were affected by the above factors. Finally, a type II analysis of variance was performed on the linear models and the obtained *P*-values were corrected with the FDR method. The content differences between infested and healthy (Xi – Xh) was plotted.

Relationships Between Root Microbial Communities and Root Chemistry

To assess the relationship between root microbial communities and root chemistry under herbivory, we applied the DIABLO (Data Integration Analysis for Biomarker discovery using Latent cOmponents) method on our data with the plant treatment (i.e., infested vs. healthy plants) defined as the grouping factor, using the scripts provided by Hervé et al. (2018) and two R packages (“mixOmics” and “RVAideMemoire”). Only metabolites or chemical elements significantly impacted by the treatment at a given time were kept for this analysis at this same given time. Bacterial and fungal genera used in this analysis were also significantly impacted by the plant treatment and bacterial count differences respected the 250/–250 threshold mentioned above. Hence, a three block-DIABLO was performed at 14 DAI on one hand, using three datasets (i.e., absolute abundance (expressed in counts of OTUs) of microbial genera, metabolites, and chemical elements) while a two block-DIABLO was performed at 42 DAI

on the other hand with two datasets: microbial genera and combined metabolites and elements. In the latter case, an analysis on three blocks was not possible using a single column dataset for chemical elements, which was why it was included in the metabolite dataset. Discrimination of the grouping factor was assessed with a permutation significance test based on cross-validation.

RESULTS

Our experiment was based on two soils of different bacterial and fungal alpha-diversities (Table S1) and phyla abundances (Figure S1).

Diversity and Structure of Microbial Communities

After 6 weeks of OSR culture in soils of different microbial diversities and 1 DAI, the bacterial and fungal alpha-diversities were similar in both root and rhizosphere compartments.

Bacteria

Root bacterial richness and diversity were significantly impacted by sampling time, initial soil microbial diversity and the interaction between time and treatment (Table 1). Diversity was also influenced by the interaction between time and initial soil microbial diversity.

At high initial soil microbial diversity, the bacterial richness (observed OTUs) of healthy plants increased with time, reaching a peak of 900 observed OTUs at 14 DAI, and then decreased at 42 DAI (Figure 2A). Herbivory reversed this trend and richness was significantly increased at 42 DAI. Richness was significantly different between healthy and infested plants both at 14 and 42 DAI. Root bacterial diversity (Shannon index) had a similar profile at high and low initial diversities whether or not plants were infested. The Shannon index was significantly lower in infested plants than in healthy plants at 14 DAI but it was higher at 42 DAI (Figure 2A).

At low initial soil microbial diversity, the bacterial richness (observed OTUs) of healthy plants did not change with time but increased significantly after herbivory (i.e., 42 DAI) (Figure 2A). At this low initial soil diversity, root bacterial diversity (Shannon index) increased in healthy plants at 14 DAI and remained stable at 42 DAI while infested plants displayed an opposite profile (Shannon index decreased at 14 DAI and increased at 42 DAI).

Rhizosphere bacterial richness and diversity were both influenced by sampling time and initial soil microbial diversity (Table 1, Figure 2A). Richness and Shannon index decreased over time in both healthy and infested plants, the latter being similar between 14 and 42 DAI. These indices showed higher values in plants grown on high soil microbial diversity than on low soil diversity.

A dbRDA confirmed that bacterial community structure was driven by compartment, time, treatment and initial soil microbial diversity (Figure 3A). Differences in bacterial community structures between healthy and infested plants were highly significant.

TABLE 1 | Statistical output of bacterial and fungal alpha-diversity indices in the root and rhizosphere compartments, associated to **Figure 2**.

Factors			Roots			Rhizosphere		
			F-value	df	P-value	F-value	df	P-value
Bacteria	Richness (observed OTUs)	Sampling time	4.61	2	0.020	11.40	2	<0.001
		Treatment	0.80	1	0.370	0.16	1	0.680
		Soil microbial diversity	32.90	1	<0.001	95.80	1	<0.001
		Soil biological replicates	1.58	2	0.220	1.57	2	0.220
		Sampling time:Treatment	19.10	2	<0.001	–	–	–
		Sampling time:Soil microbial diversity	4.07	2	0.030	–	–	–
		Treatment:Soil microbial diversity	0.35	1	0.550	–	–	–
	Diversity (Shannon index)	Sampling time	9.34	2	0.001	4.94	2	0.014
		Treatment	0.006	1	0.930	4.06	1	0.053
		Soil microbial diversity	20.80	1	<0.001	39.80	1	<0.001
		Soil biological replicates	2.04	2	0.150	1.28	2	0.290
		Sampling time:Treatment	22.10	2	<0.001	–	–	–
		Sampling time:Soil microbial diversity	1.23	2	0.300	–	–	–
		Treatment:Soil microbial diversity	0.03	1	0.850	–	–	–
Fungi	Richness (observed OTUs)	Sampling time	9.19	2	0.001	1.50	2	0.230
		Treatment	0.07	1	0.780	0.56	1	0.450
		Soil microbial diversity	4.27	1	0.050	27.70	1	<0.001
		Soil biological replicates	0.91	2	0.410	3.58	2	0.040
		Sampling time:Treatment	3.28	2	0.056	–	–	–
		Sampling time:Soil microbial diversity	0.09	2	0.900	–	–	–
		Treatment:Soil microbial diversity	1.10	1	0.300	–	–	–
	Diversity (Shannon index)	Sampling time	9.25	2	0.001	4.20	2	0.024
		Treatment	0.70	1	0.410	2.34	1	0.130
		Soil microbial diversity	4.06	1	0.056	6.38	1	0.017
		Soil biological replicates	1.24	2	0.300	1.68	2	0.200
		Sampling time:Treatment	3.71	2	0.040	–	–	–
		Sampling time:Soil microbial diversity	0.05	2	0.940	–	–	–
		Treatment:Soil microbial diversity	0.88	1	0.350	–	–	–

The above table presents the outcome of variance analyses (F test) performed on linear models, based on richness (i.e., observed OTUs) or diversity (Shannon index) of each compartment, and taking into account sampling time (1, 14, and 42 days after infestation "DAI"), treatment (healthy vs. infested plants), soil microbial diversity (high vs. low soil diversity) and 2 × 2 interactions between these factors, as well as soil replicates. F-value, degrees of freedom (df), and P-value (in bold when significant) are given for each tested factor or interaction. Dashes indicate that all interactions in the rhizosphere were non-significant and have thus been removed from the models.

Fungi

Root fungal richness was four times lower than root bacterial richness and ranged from 150 to 300 observed OTUs. It was only influenced by sampling time (**Table 1**). The richness of healthy and infested plants at both soil diversities similarly increased over time and reached 250 OTUs at 42 DAI (**Figure 2B**).

Root fungal diversity (Shannon index) was impacted by time and by the interaction between time and treatment but not by soil microbial diversity (**Table 1**). Both richness of healthy and infested plants increased over time but the Shannon index was higher in infested plants at 14 DAI, hence during the peak of herbivory (**Figure 2B**).

Rhizosphere fungal richness was similar to root richness, with 250 to 350 observed OTUs, and it did not vary as much as bacterial richness. Rhizosphere fungal richness was impacted by initial soil microbial diversity and by the soil biological replicates (**Table 1**). Richnesses of healthy and infested plants remained

similarly stable over time (**Figure 2B**). Richness was greater in plants grown on high soil microbial diversity.

Rhizosphere fungal diversity was influenced by both sampling time and initial soil microbial diversity (**Table 1**). The Shannon index of healthy and infested plants increased similarly over time and diversity was greater in plants grown on high soil microbial diversity (**Figure 2B**).

Fungal community structure drivers were compartment, time, and treatment, which were highly significant, as well as initial soil microbial diversity (dbRDA, **Figure 3B**).

Identification of Microbial Phyla and Genera Associated With Herbivory and Initial Soil Microbial Diversity

Relative Abundance of Microbial Phyla

The roots (**Figure 4A**) and rhizosphere (**Figure 4B**) contained four major bacterial phyla, of which *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* were the most abundant. The root bacterial

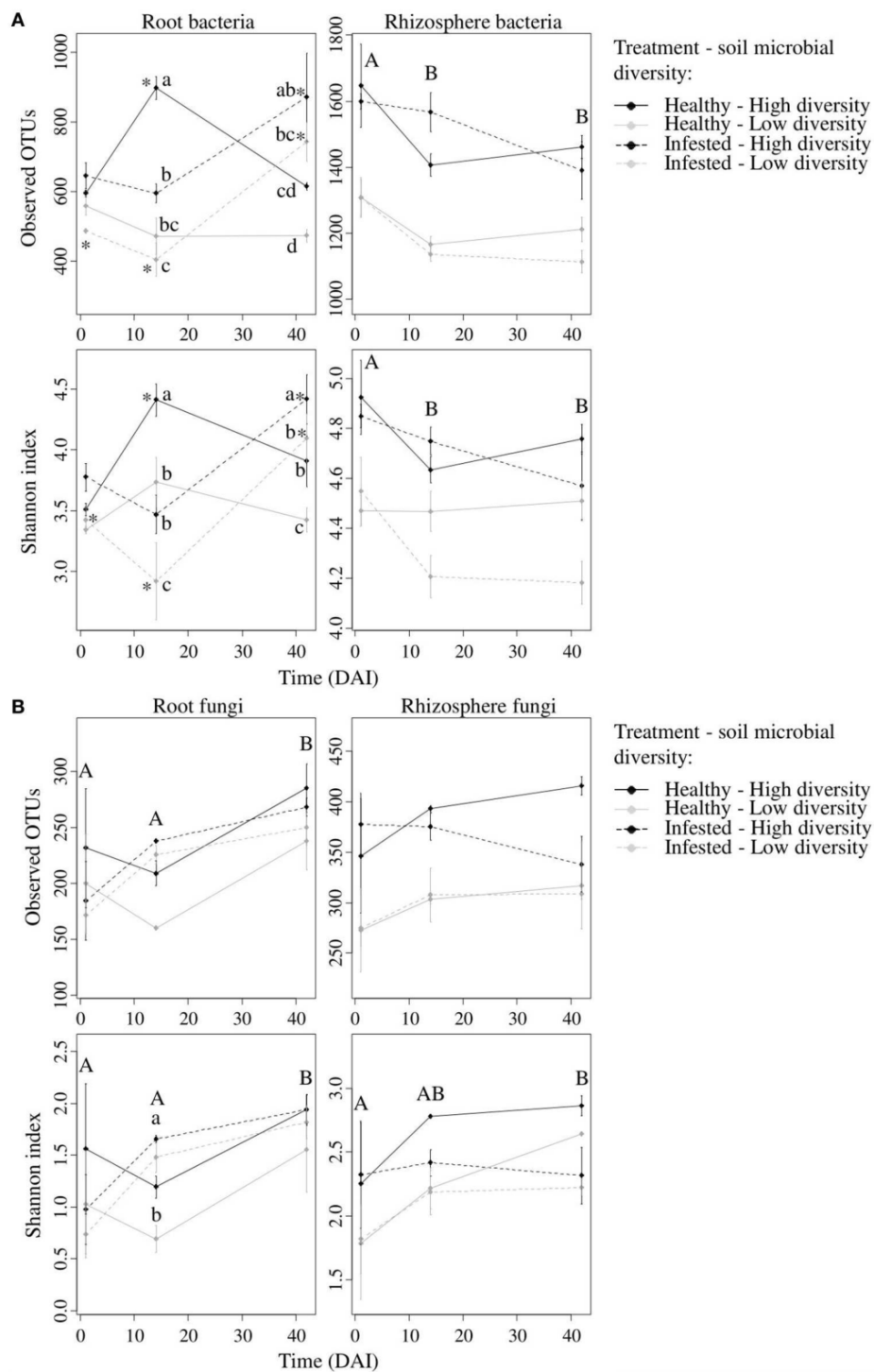
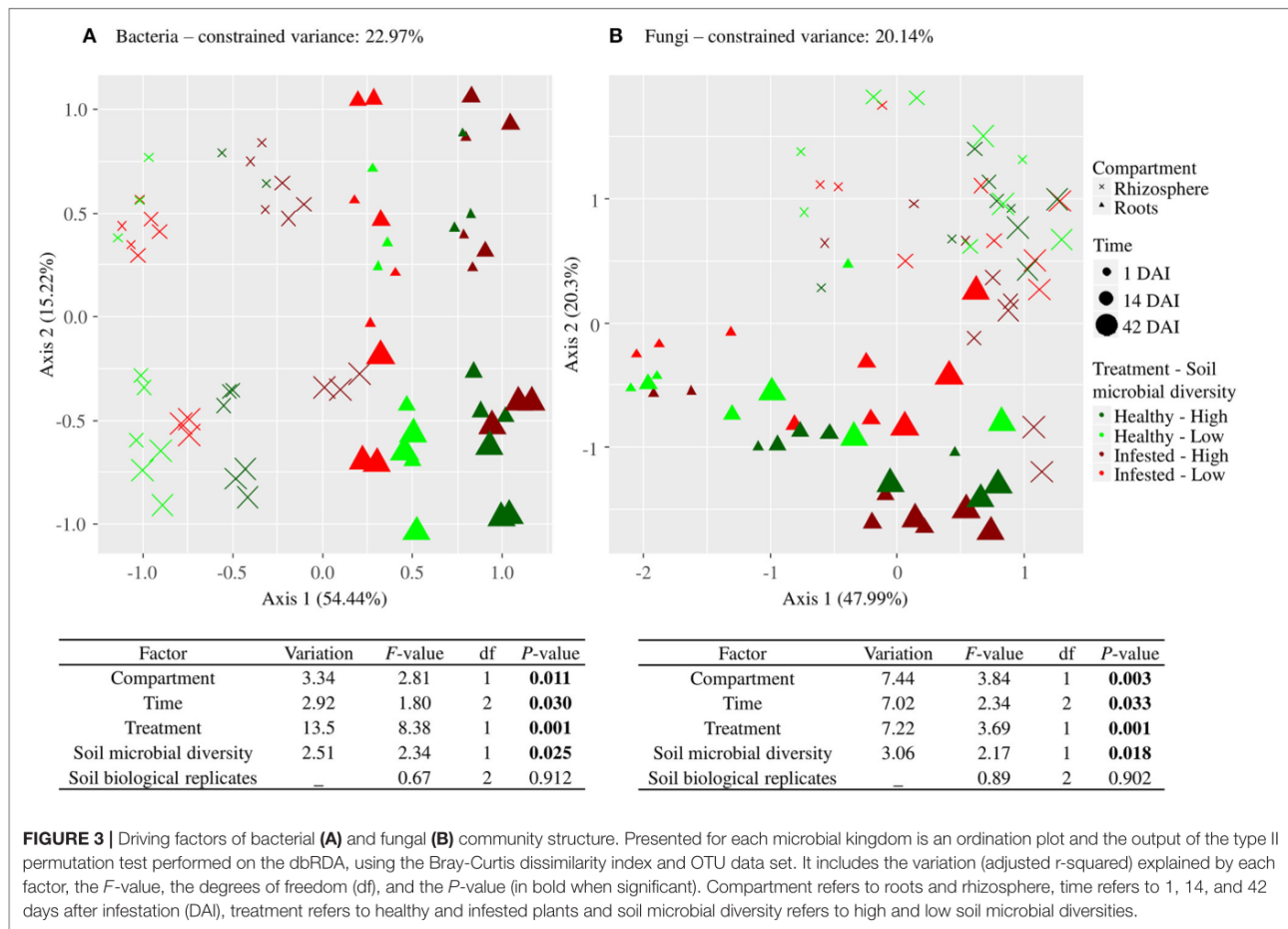


FIGURE 2 | Bacterial and fungal alpha-diversity indices in the root and rhizosphere compartments. Mean value (\pm se) of bacterial **(A)** and fungal **(B)** richness (i.e., Observed OTUs) and diversity (i.e., Shannon index) for the root (left column) and rhizosphere (right column) compartments at 1, 14, and 42 days after infestation (DAI). In the root compartment, lowercase letters represent significant differences between modalities at one given time while asterisks represent significant differences between times for one given modality. Regarding the rhizosphere compartment, capital letters represent significant differences between times for all modalities.



phyla were mainly influenced by infestation but not at 1 DAI. Significant differences occurred during the peak of herbivory at 14 DAI: the phylum of γ -Proteobacteria increased under herbivory, while the phyla of α -, δ -Proteobacteria, Actinobacteria, Bacteroidetes, and “Others” decreased. At 42 DAI, infestation still influenced the phyla of γ -Proteobacteria, Actinobacteria and “Others.” Initial soil microbial diversity impacted β -Proteobacteria at 14 and 42 DAI with a larger abundance in high diversity-infested plants while on the contrary Actinobacteria was more abundant in low diversity-infested plants at 42 DAI.

The bacterial phyla of the rhizosphere (Figure 4B) were also influenced by infestation as well as the initial soil microbial diversity and their abundances were in the same range as in the roots. Overall, the phyla of Actinobacteria, β -, δ -, γ -Proteobacteria, and “Others” differed between high and low microbial diversities at the different times. Infestation started to impact phyla at 14 DAI, with more γ -Proteobacteria and less α -Proteobacteria in infested plants. At 42 DAI, the phyla of α -Proteobacteria and Firmicutes were still affected by infestation and more abundant in infested plants.

In the roots and the rhizosphere, there were four major fungal phyla among which Ascomycota (divided into three

sub-phyla), Basidiomycota and Chytridiomycota were the most abundant. Relative abundances of root fungal phyla (Figure 4C) were very variable. At 1 DAI, there was no difference between modalities. Pezizomycotina and Basidiomycota abundances increased in infested plants at 14 and 42 DAI respectively while Taphrinomycotina abundance decreased at 42 DAI. The phyla of Basidiomycota and Chytridiomycota were influenced by the soil microbial diversity at 42 DAI in infested plants.

Fungal phyla (Figure 4D) seemed to vary less in the rhizosphere than in the roots but proportions of Chytridiomycota seemed to decrease in favor of Basidiomycota, Pezizomycotina, and Saccharomycotina. There was no effect of herbivory on fungal phyla at 1 and 14 DAI, but Chytridiomycota abundance decreased in infested plants at 42 DAI. The phyla of Blastocladiomycota and “Others” were lightly influenced by the initial soil microbial diversity, with larger abundances in plants of high diversity.

Absolute Abundance of Microbial Genera

A total of 2,031 bacterial genera were detected in the roots and in the rhizosphere.

The root compartment presented 74 bacterial genera from nine different phyla, which varied significantly following

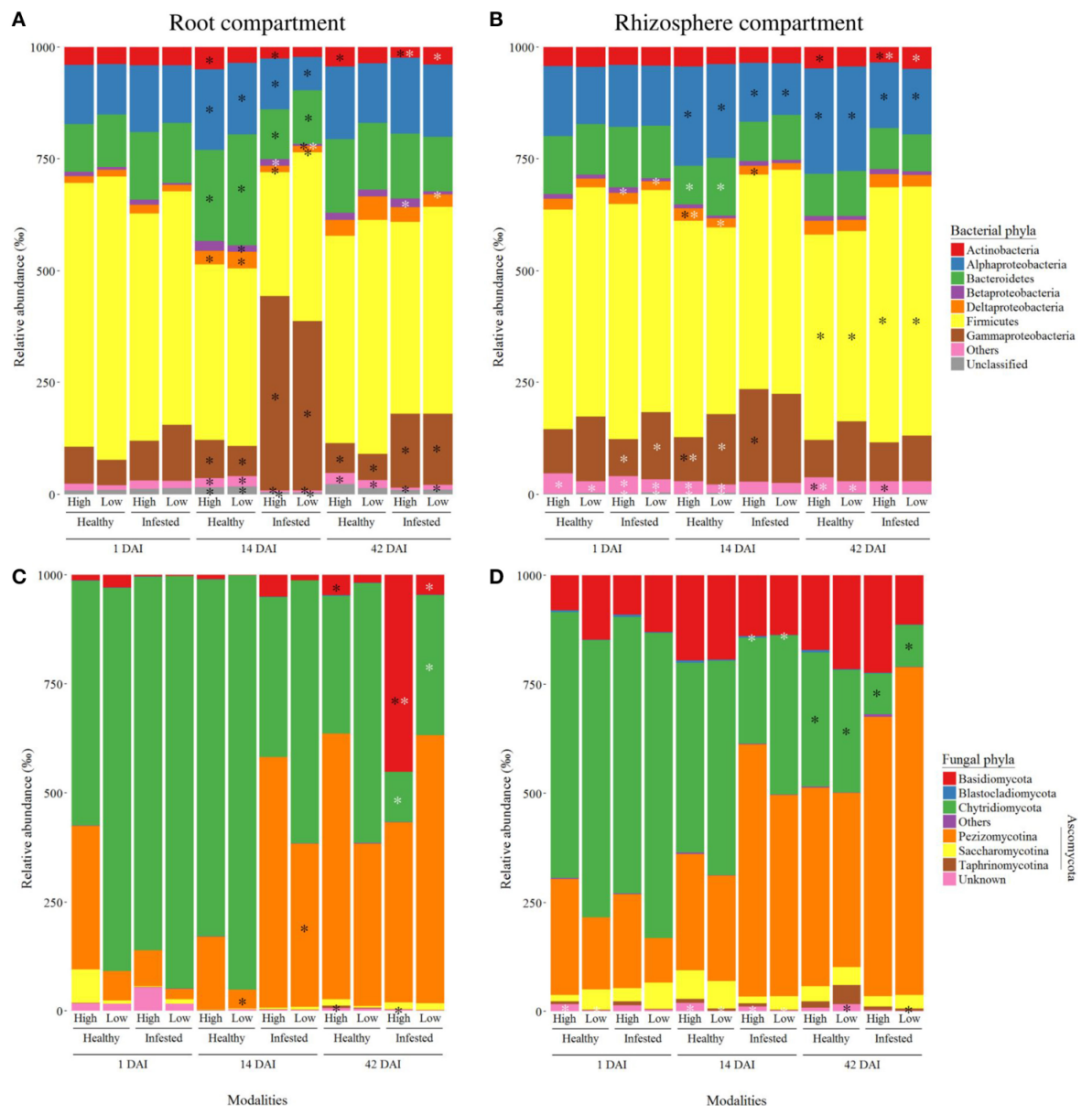
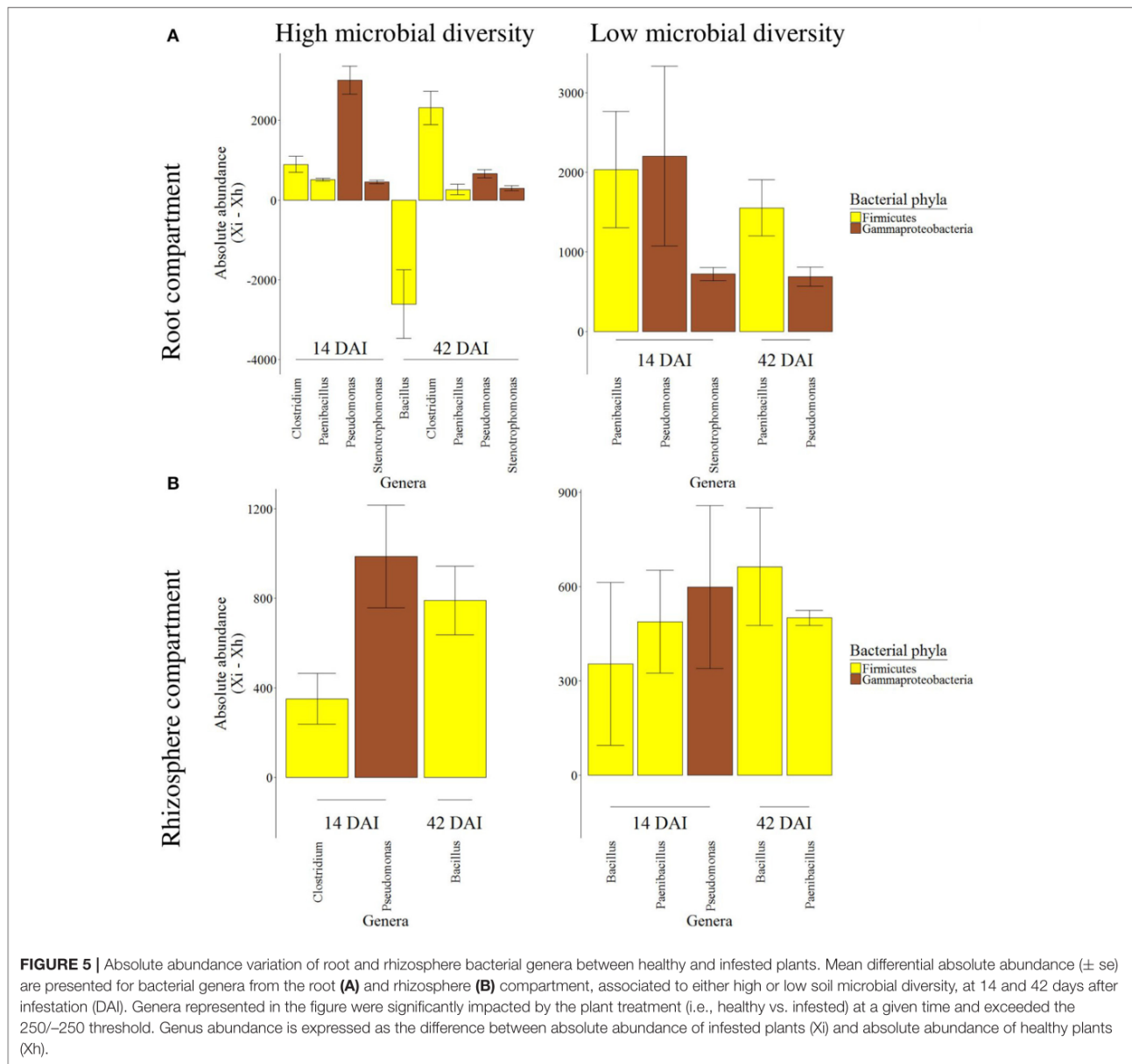


FIGURE 4 | Dominant bacterial and fungal phyla detected in the roots and rhizosphere. Mean relative abundance are presented for bacterial (A,B) and fungal (C,D) phyla from the root (A,C), and rhizosphere (B,D) compartments. All modalities are represented, taking into account the sampling time (1, 14, and 42 days after infestation), the treatment (healthy and infested plants), and initial soil microbial diversity (high and low levels). Black asterisks indicate significant differences between healthy and infested plants at a given time and soil microbial diversity, while white asterisks indicate significant differences between soil microbial diversities at a given time and for a given treatment.

herbivory. Most of these genera showed a different abundance between healthy and infested plants at 14 and/or 42 DAI but not at 1 DAI (Table S2). Out of these 74 genera, *Bacillus*, *Clostridium*, *Paenibacillus*, *Pseudomonas*, and *Stenotrophomonas* were the most abundant genera influenced by herbivory (Figure 5A). *Bacillus* decreased in infested plants while the other four genera increased and none of them returned to an abundance similar to the one detected in healthy plants at 42 DAI. Abundance varied with the initial soil microbial diversity. At low soil diversity, the increase of *Paenibacillus* abundance following herbivory was

greater than at high soil diversity while that of the other genera were similar between both diversities.

In the rhizosphere compartment, fewer bacterial genera (46 genera from 9 phyla) were impacted by the infestation at 14 and/or 42 DAI (Table S3). Compared to the roots, the range of bacterial absolute abundance in the rhizosphere was lower. This time, only *Bacillus*, *Clostridium*, *Paenibacillus*, and *Pseudomonas* were the most abundant genera influenced and increased by herbivory (Figure 5B). As previously shown in the roots, the rhizosphere was also characterized by a change of range in the



differential abundance associated to the initial soil microbial diversity, where the range was lower at low diversity.

A total of 2,593 fungal genera were detected in the roots and in the rhizosphere. The fungal genera varied as much as their phyla (Table S4). In the roots, four out of the five herbivory-influenced genera were more abundant in infested plants, including *Ajellomyces* and *Filobasidiella* from the subphylum of *Pezizomycotina* and the phylum of *Basidiomycota* respectively. No herbivory-influenced genus was significantly impacted at low microbial diversity in the roots. In the rhizosphere, eight genera were influenced by infestation, *Torulaspora* and *Tuber* being more abundant in infested plants at 1 and 42 DAI respectively (Table S4).

Root Compounds Modulated by Herbivory Primary and Secondary Metabolites

Metabolomic profile of OSR roots was significantly influenced by the treatment at 1, 14, and 42 DAI (Table 2) where infested and healthy plants showed two different profiles, associated to metabolite variations (Figure S2). Their profiles were also impacted by the initial soil microbial diversity but only at 14 DAI. The constrained variance (i.e., part of the variance explained by our variables) had the highest value (73%) at 14 DAI.

Overall, 33 metabolites (17 AAs, 9 CPOs, and 7 GSLs presented in Figures 6A–C respectively) were significantly affected by infestation at one or several moments of the plant-insect interaction and 2 metabolites were impacted by the

TABLE 2 | Statistical output of multivariate analysis performed on root metabolites and elements.

Compounds	Time	Total variance (%)	Constrained variance (%)	Factor	RDA test
Metabolites	1 DAI	Constrained = 67.28 Unconstrained = 32.72	Axis 1 = 67.96 Axis 2 = 16.73	Treatment	$F = 8.11$; $df = 1$; $P = \mathbf{0.002}$
				Soil microbial diversity	$F = 0.97$; $df = 1$; $P = 0.375$
				Soil biological replicates	$F = 1.07$; $df = 2$; $P = 0.354$
				Interaction Treatment-Soil microbial diversity	$F = 1.09$; $df = 1$; $P = 0.313$
	14 DAI	Constrained = 73.49 Unconstrained = 26.51	Axis 1 = 66.70 Axis 2 = 14.45	Treatment	$F = 9.95$; $df = 1$; $P = \mathbf{0.001}$
				Soil microbial diversity	$F = 3.22$; $df = 1$; $P = \mathbf{0.039}$
				Soil biological replicates	$F = 1.28$; $df = 2$; $P = 0.247$
				Interaction Treatment-Soil microbial diversity	$F = 0.87$; $df = 1$; $P = 0.463$
	42 DAI	Constrained = 61.18 Unconstrained = 38.82	Axis 1 = 52.32 Axis 2 = 23.88	Treatment	$F = 4.48$; $df = 1$; $P = \mathbf{0.002}$
				Soil microbial diversity	$F = 1.29$; $df = 1$; $P = 0.271$
				Soil biological replicates	$F = 1.45$; $df = 2$; $P = 0.184$
				Interaction Treatment-Soil microbial diversity	$F = 0.77$; $df = 1$; $P = 0.602$
Elements	1 DAI	Constrained = 54.38 Unconstrained = 45.62	Axis 1 = 46.08 Axis 2 = 39.89	Treatment	$F = 2.66$, $Df = 1$, $P = 0.055$
				Soil microbial diversity	$F = 2.00$, $Df = 1$, $P = 0.122$
				Soil biological replicates	$F = 0.80$, $Df = 2$, $P = 0.610$
				Interaction Treatment-Soil microbial diversity	$F = 0.87$, $Df = 1$, $P = 0.488$
	14 DAI	Constrained = 61.26 Unconstrained = 38.74	Axis 1 = 61.20 Axis 2 = 23.68	Treatment	$F = 5.20$, $Df = 1$, $P = \mathbf{0.006}$
				Soil microbial diversity	$F = 2.02$, $Df = 1$, $P = 0.105$
				Soil biological replicates	$F = 0.50$, $Df = 2$, $P = 0.897$
				Interaction Treatment-Soil microbial diversity	$F = 1.24$, $Df = 1$, $P = 0.301$
	42 DAI	Constrained = 55.99 Unconstrained = 44.01	Axis 1 = 55.54 Axis 2 = 27.07	Treatment	$F = 2.68$, $Df = 1$, $P = \mathbf{0.045}$
				Soil microbial diversity	$F = 1.16$, $Df = 1$, $P = 0.298$
				Soil biological replicates	$F = 0.73$, $Df = 2$, $P = 0.650$
				Interaction Treatment-Soil microbial diversity	$F = 2.31$, $Df = 1$, $P = 0.079$

The above table presents the outcome of permutation test performed on RDA, based on root metabolite and element contents, and taking into account the treatment (healthy vs. infested plants), soil microbial diversity (high vs. low soil diversity), soil biological replicates and the interaction between treatment and diversity. Total and constrained variances, F -value, degrees of freedom (df), and P -value (in bold when significant) are given for the tested factors and interaction, at each sampling time (1, 14, and 42 days after infestation "DAI").

soil microbial diversity (Table S5). Many of them differed at 1 and/or 14 DAI and most of these 33 metabolites were less abundant in infested plants except 2 AAs (β -alanine, SMCSO), 2 CPOs (trehalose, glycerate), and 2 GSLs (glucobrassicin, neoglucobrassicin), which production increased. Twenty-three of these metabolites reached back a stable state, close to the profile of healthy plants at 42 DAI. All GSLs varied due to infestation but six were still influenced at 42 DAI.

Chemical Elements

Elemental profiles of OSR roots were only impacted by the treatment at 14 and 42 DAI (Table 2, Figure S3). At 14 DAI, the profile of infested plants was highly modified compared to healthy plants. As for metabolites, the constrained variance was the highest at 14 DAI (61%).

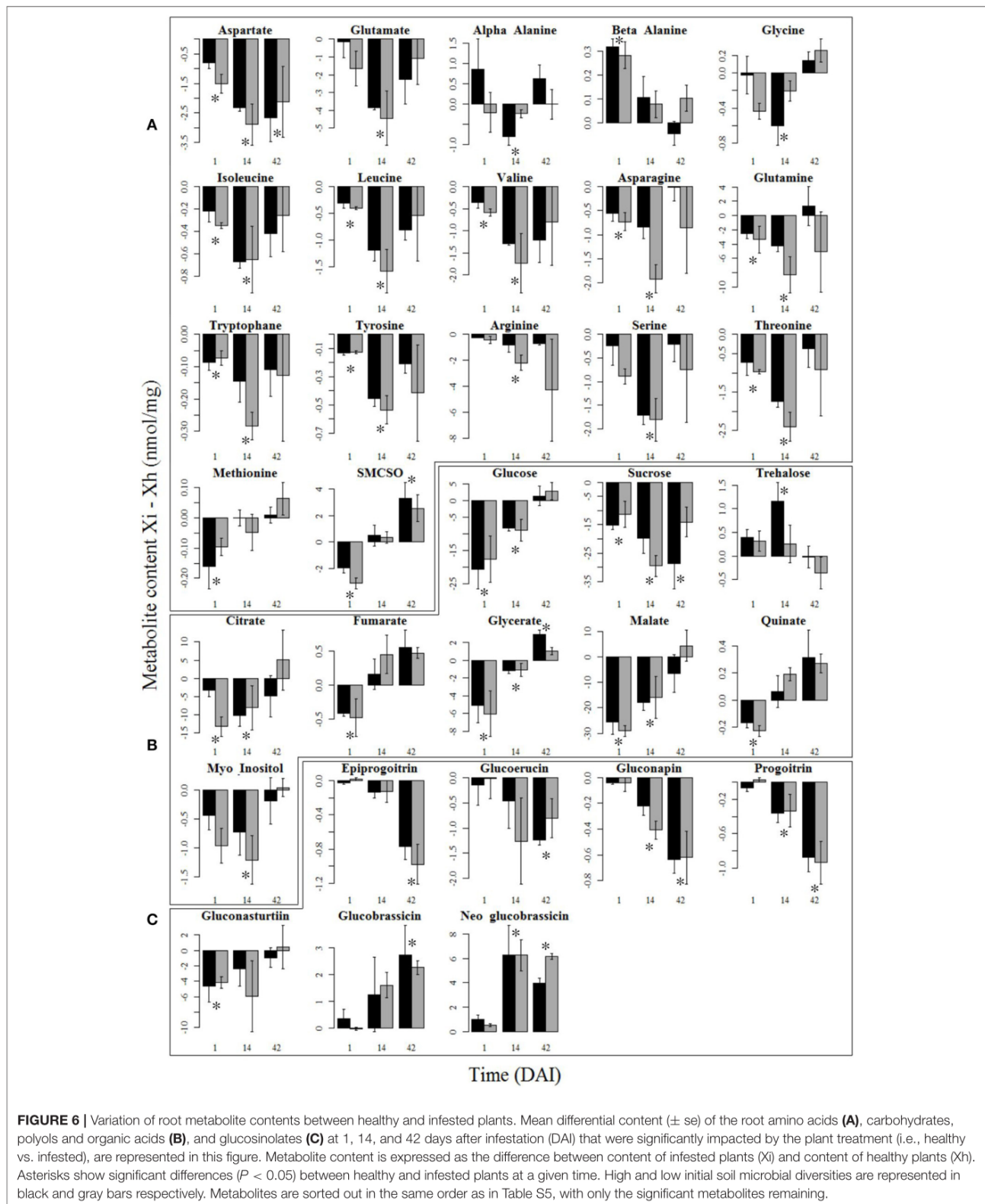
Only 8 chemical elements (4 macroelements, 3 microelements, and 1 heavy metal) were significantly impacted by the infestation, while soil microbial diversity had no effect on chemical element content (Figure 7, Table S6). Six elements (Mg, K, Fe, Na, V, Cd) decreased in infested plants at 14 DAI while N content increased. All of these elements reached a state similar to healthy plants at 42

DAI. Sulfur also increased in infested plants, but only at 42 DAI.

Relationships Between Root Compounds and Microbial Communities

At the peak of herbivory (i.e., 14 DAI), the DIABLO method confirmed the significant differences between infested and healthy plants ($CER = 0$, $P = 0.002$) with the three score plots showing similar patterns (Figure 8). The axis 1 of microbiome data was positively correlated to axes 1 of metabolite and element data ($r = 0.86$ and 0.95 , respectively) while metabolite data and element data were also positively correlated ($r = 0.84$). Infested plants seemed to be associated to (i) four bacterial genera (*Clostridium*, *Paenibacillus*, *Pseudomonas*, and *Stenotrophomonas*) and one fungal genus (*Torulaspora*), that were overexpressed, (ii) trehalose (CPO) and neoglucobrassicin (GSL), also overexpressed, (iii) nitrogen but to a lesser extent. The remaining AAs, CPOs, GSLs and seven elements were underexpressed in infested plants.

At 42 DAI, the discrimination between infested and healthy plants was also significant ($CER = 0$, $P = 0.002$) and axis 1 of microbiome data was highly positively correlated to axis 1 of combined metabolite and element data ($r = 0.95$), confirmed



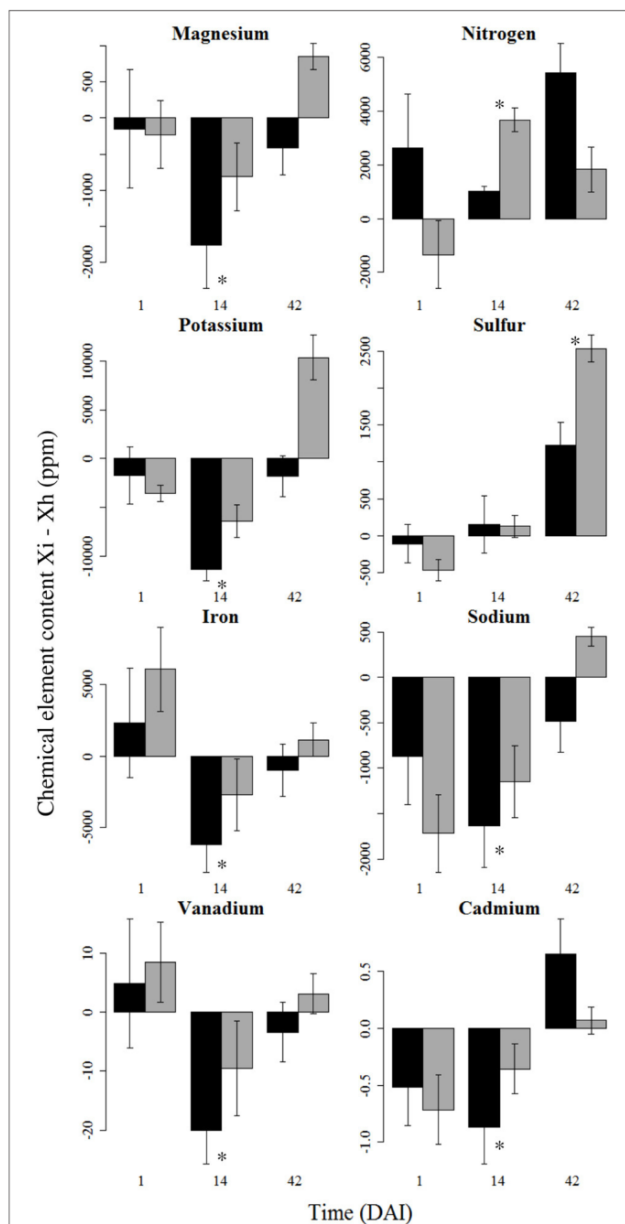


FIGURE 7 | Variation of root chemical element content between healthy and infested plants. Mean differential content (\pm se) of the macro- (Mg, N, K, S), microelements (Fe, Na, V), and heavy metal (Cd) at 1, 14, and 42 days after infestation (DAI) that were significantly impacted by the plant treatment (i.e., healthy vs. infested), are represented in this figure. Chemical element content is expressed as the difference between content of infested plants (Xi) and content of healthy plants (Xh). Asterisks show significant differences ($P < 0.05$) between healthy and infested plants at a given time. High and low initial soil microbial diversities are represented in black and gray bars respectively. Chemical elements are sorted out in the same order as in Table S6, with only the significant chemical elements remaining.

by similar patterns on the two score plots (Figure 9). At the end of herbivory, infested plants seemed to be associated to (i) four bacterial genera (*Clostridium*, *Paenibacillus*, *Pseudomonas*,

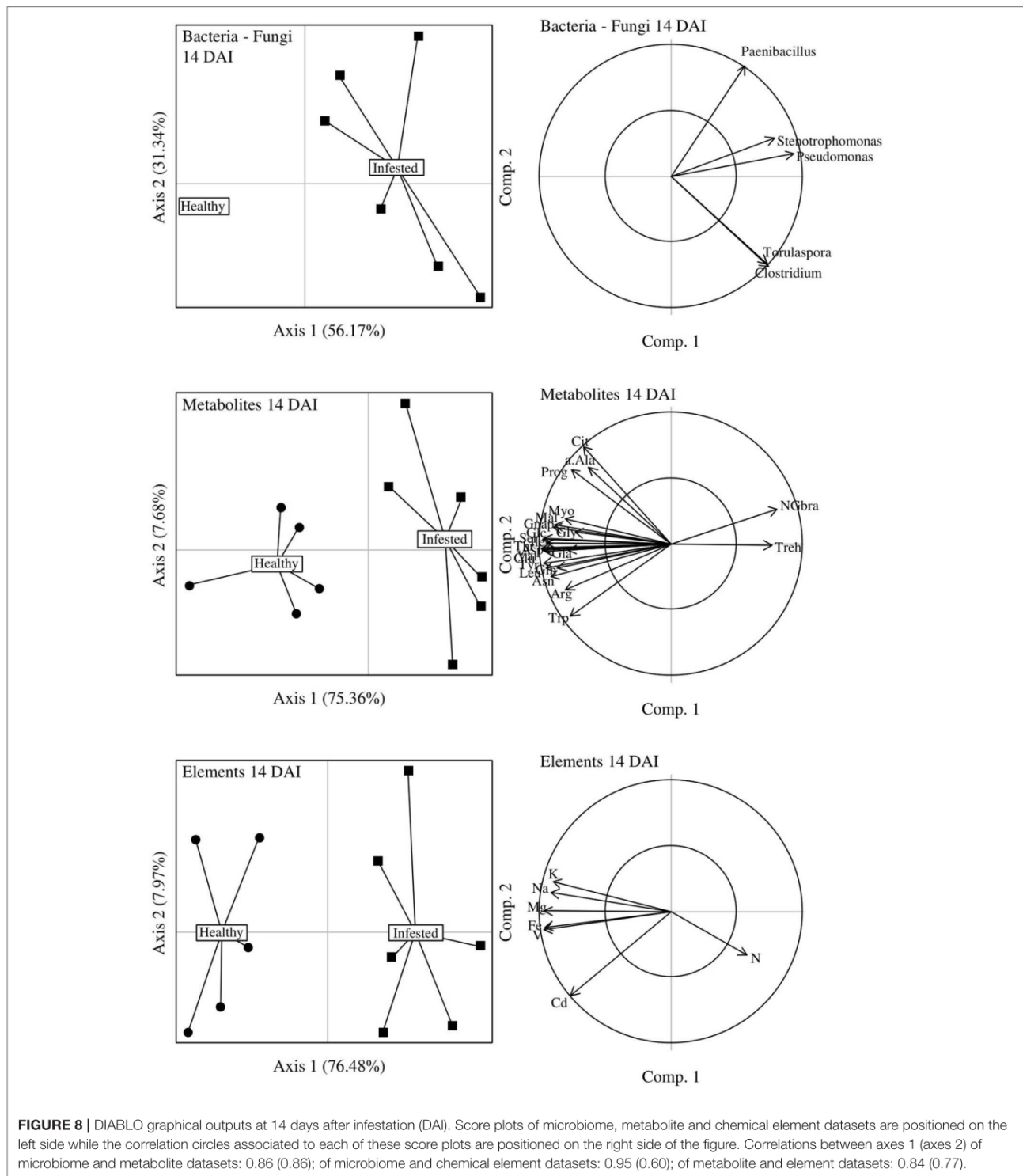
Stenotrophomonas) and three fungal genera (*Ajellomyces*, *Filobasidiella*, *Torulaspora*), which were overexpressed and (ii) SMCSO (AA), glycerate (CPO), glucobrassicin and neoglucobrassicin (GSLs), and sulfur. Conversely, one bacterial and one fungal genus, as well as four metabolites were underexpressed in these infested plants.

DISCUSSION

Our study showed that infestation of OSR by a belowground herbivore increased root bacterial alpha-diversity, while it had no effect on rhizosphere bacteria and very little on fungi. Interestingly, herbivory was associated with an increase of γ -Proteobacteria and Firmicutes (and five of their most dominant affiliated genera: *Pseudomonas*, *Stenotrophomonas* and *Bacillus*, *Clostridium*, *Paenibacillus* respectively) as well as *Ajellomyces* and *Filobasidiella* from the fungal phyla of *Ascomycota* and *Basidiomycota*. Trehalose, sulfur-containing metabolites and sulfur contents increased in infested plants and could explain the variations observed in bacterial phyla and genera. Herbivory seemed to have only a short-term negative effect on the richness and diversity of the microbial communities, which were both restored when the perturbation ended. The chemical composition of roots matched this restoration. Initial soil microbial diversity itself had little impact on microbial communities of the root and its rhizosphere and did not significantly influence root chemistry.

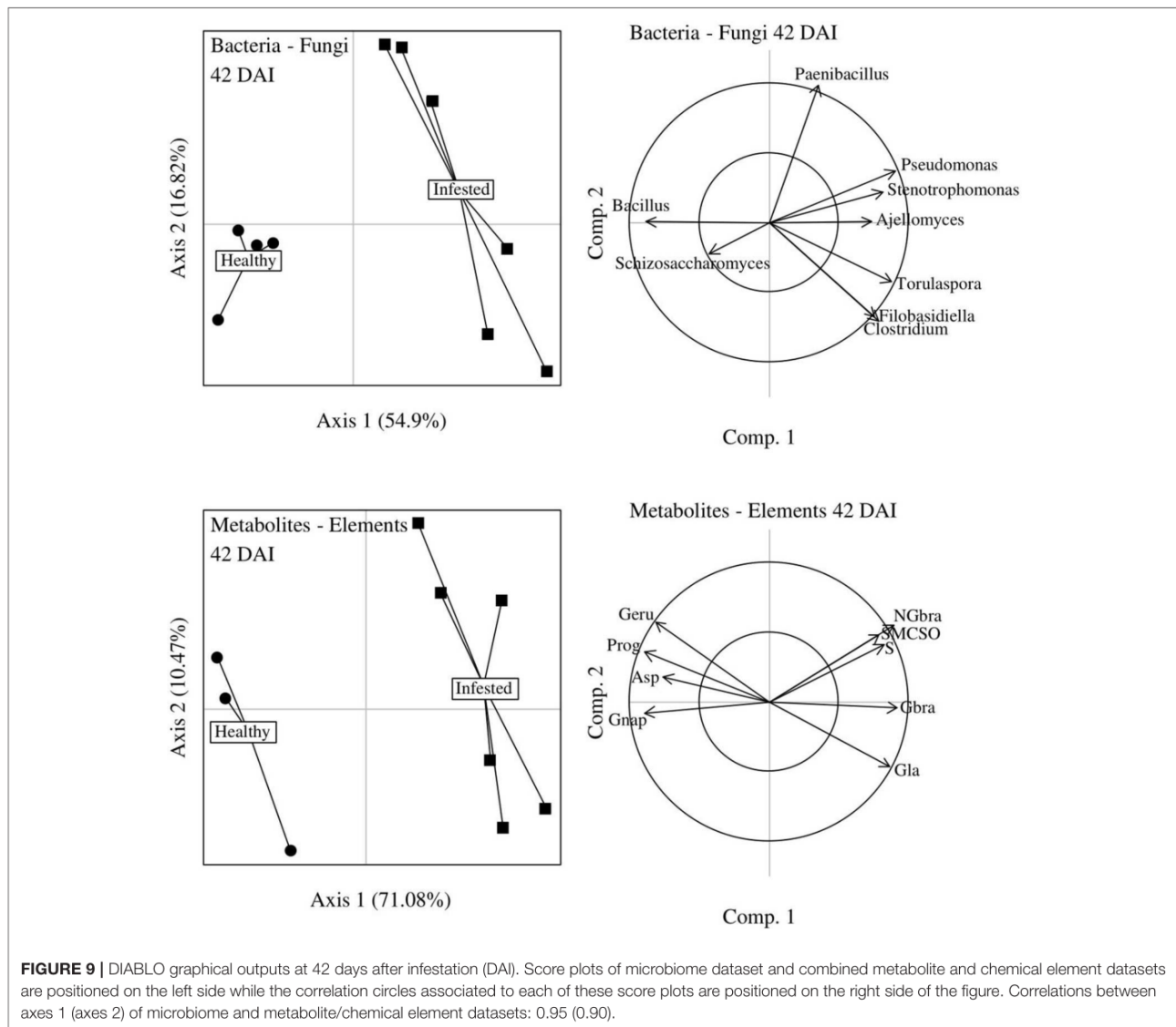
Herbivory Influences Microbial Community Diversity and Composition

Our study showed that root herbivory decreased bacterial richness and diversity in roots at the peak of herbivory but globally increased both at the end of herbivory. Opposite trends were found in grassland plant communities, where plant diversity decreased after the end of herbivory (e.g., Collins et al., 1998; Pucheta et al., 1998) but microbial communities may be hard to compare with plant communities. Further from the roots, bacterial and fungal communities of the rhizosphere were respectively not affected and lightly affected by herbivory in our study. In other studies on the putative influence of herbivory on bacterial communities, herbivory marginally increased the abundance of soil nitrifying bacteria and archaea (Le Roux et al., 2008) and, (in contradiction with our own results) Kong et al. (2016) found that white fly herbivory significantly decreased bacterial richness in the rhizosphere of pepper. Differences between these two earlier studies may be due to differences in the biological models studied but also to the nature and length of the perturbation and type of experiments. Compared to bacteria, we observed that fungal communities were very variable and only lightly influenced by herbivory. These results are consistent with previous studies showing that fungi are quite resistant to herbivory, such as leaf mining in *Ageratina altissima* (Asteraceae), which did not influence the communities of endophytes colonizing leaves (Christian et al., 2016).



Roots and rhizosphere microbial communities of healthy OSR were mainly composed of bacterial taxa *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* (more specifically

of *Bacillus*, *Paenibacillus*, and *Pseudomonas* at the genera level), while fungal taxa were mainly *Ascomycota*, *Chytridiomycota* and *Basidiomycota*. Our results on bacteria are in line with



those of Bulgarelli et al. (2012) and Bodenhausen et al. (2013) in *Arabidopsis thaliana*. Regarding fungi, *Ascomycota* and *Basidiomycota* accounted for 96% of the community in *Microthlaspi* spp. populations, another brassicaceous plant (Glynou et al., 2018). A recent study on OSR showed that *Proteobacteria* and *Actinobacteria* as well as *Ascomycota* and *Basidiomycota* were the most abundant bacterial and fungal phyla respectively in the roots and rhizosphere, however with different dominant genera (Gkarmiri et al., 2017). While the phylum of *Chytridiomycota* was only present in roots in the previous study, it was part of the rhizosphere composition in ours. These differences in phyla and genera might be due to the use of isotopic labeling by Gkarmiri et al. (2017), representing only the active portion (i.e., assimilating labeled photosynthetates) of the plant communities. Furthermore, many drivers have been shown to shape plant microbial communities:

plant root exudates (Turner et al., 2013; Tsunoda and van Dam, 2017); plant development (de Campos et al., 2013; Chaparro et al., 2014; Wagner et al., 2016); accession (Micallef et al., 2009); genotype (Wagner et al., 2016); species (Miethling et al., 2000); soil (Vandenkoornhuyse et al., 2015); and agricultural management practices (Hartmann et al., 2015; Rathore et al., 2017). It is therefore not surprising to find some differences in our study.

Root herbivory increased the abundance of γ -Proteobacteria in the roots and rhizosphere, and that of Firmicutes in the rhizosphere but it decreased abundance of α -Proteobacteria in both compartments and δ -Proteobacteria and *Bacteroidetes* in the roots only. These changes were accompanied by an increased abundance of *Bacillus*, *Paenibacillus*, *Pseudomonas*, and *Stenotrophomonas*. However, some of these phyla in infested plants at 42 DAI reached

back a stable state similar to that found in healthy plants at 42 DAI, hence highlighting the resilience of microbial communities after the end of insect herbivory. These results are consistent with those of Kong et al. (2016) on whitefly herbivory on pepper showing an increase in the abundance of *γ-Proteobacteria* and *Stenotrophomonas* in the rhizosphere and with those of Kim et al. (2016) where aphid herbivory increased the abundance of *Paenibacillus*. According to Card et al. (2015), *Bacillus*, *Paenibacillus*, *Pseudomonas*, and *Stenotrophomas* are either inhibitors or antagonists of plant-pathogenic bacteria or fungi, while *Bacillus* and *Paenibacillus* are known to be also entomopathogenous (Monnerat et al., 2009; Grady et al., 2016; Zhao et al., 2017).

Herbivory also affected fungal phyla and genera but to a lesser extent than bacterial taxa: it decreased the abundance of *Chytridiomycota* in the rhizosphere and increased the abundance of *Ajellomyces* (slightly) and *Filobasidiella* (strongly) in the roots. Tkacz et al. (2015) demonstrated an increase of a *Chytridiomycota* in the rhizosphere of *Brassica rapa* over time, while this phylum progressively colonized the roots during plant development, to finally dominate fungal communities (Lebreton, personal communication). Most fungi are oligotrophic and grow slowly, with limited carbon sources (Ho et al., 2017). The lesser effect of herbivory on fungal communities observed in our study might be due to the timeframe of our experiment, probably too short compared to the growth rate of fungi, which is based on their utilization of complex trophic resources.

Herbivory Influences Root Metabolite and Chemical Element Composition

Herbivory tended to decrease root AAs and CPOs, which is consistent with the results of Hopkins et al. (1999), van Leur et al. (2008), and Lachaise et al. (2017). Herbivory increased glycerate and trehalose, a sugar assumed to play a role in plant defenses against aphid infestation (Singh et al., 2011). Sucrose was not affected by herbivory, contrary to the study of Pierre et al. (2012). S-methyl cysteine sulfoxide (SMCSO), a toxic compound detectable in brassicaceous crops (Edmands et al., 2013) was increased by herbivory. We therefore hypothesize that both SMCSO and trehalose might play a role in OSR defense against root herbivory.

Our study showed that herbivory also modulated GSLs by increasing two indolyl (glucobrassicin and neoglucobrassicin) but decreasing four aliphatic and one aromatic GSLs consistently with van Dam and Raaijmakers (2006). This is also coherent with the same decrease of total GSL contents found by van Dam and Raaijmakers (2006) and Lachaise et al. (2017). A recent study demonstrated the detrimental effect of glucobrassicin on aboveground generalist and specialist herbivores: a lower larval weight and faster development time associated with an increased mortality (Santolamazza-Carbone et al., 2017). However, in another study, aliphatic GSLs affected larval weight in generalists but not in specialist insects (Arany et al., 2008). Glucosinolates are generally considered as

defensive compounds against herbivores but their impact on insect life history traits appear very species specific and their influence on microbial communities is difficult to predict.

Herbivory increased sulfur and nitrogen contents. The increase of sulfur content in root tissues probably corresponded to higher amounts of indolyl GSLs (glucobrassicin and neoglucobrassicin) and SMCSO, which are all sulfur-containing metabolites. The increase in nitrogen could be linked to insect frass (Kagata and Ohgushi, 2011). These authors demonstrated that there was more nitrogen excreted by larva than ingested and they suggested that the excess of nitrogen might originate from the plant organic nitrogen (e.g., AAs or proteins), which could not be digested by the insect. These observations could explain our results, especially since the nitrogen increased occurred only at 14 DAI, the peak of herbivory when the CRF was still at the larval stage.

Relationship Between Bacterial Genera and Root Chemistry in Response to Herbivory: Focus on Dominant Genera and Compounds Increased by Herbivory

Root herbivory—obviously—generates root degradation, which creates habitat spatial heterogeneity and as a consequence could modify microbial communities. Changes in microbial diversity and composition following herbivory is often hypothesized to be based on modifications of the plant chemistry, such as plant exudates (Kim et al., 2016; Kong et al., 2016). However, variations in root metabolite and rhizodeposits occur during the plant's life cycle. In addition, bacteria and fungi are known to differ in their ability to use organic compounds (Boer et al., 2005). In our study, we focused on the vegetative stage of OSR, a stage when fungal communities show only limited changes (Mougel et al., 2006).

Increased trehalose following herbivory was associated with an overexpression of *Stenotrophomonas* and *Pseudomonas*. *Stenotrophomonas* appears to produce trehalose as an osmoprotective substance (Wolf, 2002) while *Pseudomonas* is able to use this compound as a carbon source for its growth (De La Fuente et al., 2007) as well as other sugars found in root exudates, a trait that makes this genus quite competitive in microbial communities (Lugtenberg et al., 1999). A modification of plant microbial communities mediated by plant chemistry was also found in Kim et al. (2016) where bacteria such as *Paenibacillus* were able to grow on medium-based root exudates, which came from aphid-infested plants. Moreover, glycerate was also associated with microbial changes under herbivory. This could be explained by the fact that glycerate-derived compounds are usually accumulated by microorganisms under abiotic stress and this process could be similar for biotic stress (see review on abiotic stress by Empadinhas and da Costa, 2011). We suggest that the root chemistry disturbed by CRF herbivory might be beneficial to colonization of microorganisms such as *Pseudomonas*, exhibiting

competitive traits to exploit trophic resources (Ho et al., 2017).

Following herbivory, enhancement of sulfur-containing compounds (i.e., SMCs, glucobrassicin, neoglucobrassicin, sulfur) was associated to an overexpression of *Bacillus*, *Paenibacillus*, *Pseudomonas*, and *Stenotrophomonas*. Aziz et al. (2016) demonstrated that plant biomass loss caused by aboveground herbivory was lessened in presence of *Bacillus*. This decrease seemed mediated by an increase of indolyl GSLs. The fact that these bacteria are associated to sulfur-containing compounds might be linked to their potential role in the sulfur cycle. *Pseudomonas* for example dominates the arylsulfatase bacterial communities in the OSR rhizosphere (Cregut et al., 2009) and this enzyme (produced by microorganisms in order to mineralize organic sulfur) is present both in the roots and in the rhizosphere of this plant (Knauff et al., 2003). A plant undergoing herbivory could send external signals to recruit bacteria, which could stimulate the synthesis of plant defense compounds (e.g., GSLs). An increase of trehalose in the roots and rhizosphere via root exudates (Paterson et al., 2007) could represent an external signal, attracting beneficial symbionts and promoting bacterial growth. These bacteria could then enhance enzymatic activities (e.g., arylsulfatase) allowing sulfur mineralization and stimulation of GSL production.

Following herbivory, nitrogen was also associated with microorganisms but to a lesser extent. Nitrogen is a limiting factor for plant growth because of its low bioavailability in the soil, since eukaryotes are not able to fix atmospheric nitrogen (Grady et al., 2016). To have access to nitrogen sources, plants rely on nitrogen-fixing microorganisms (i.e., diazotrophs). *Clostridium* and *Paenibacillus* are free-living diazotrophic species (Choudhary and Varma, 2016; Grady et al., 2016) while a nitrogen fixing trait was recently discovered in the genome of a *Pseudomonas* species (Yan et al., 2008). We suggest that plants stressed by herbivory might be able to select better microorganisms (i.e., in terms of fitness and cost in nutrients, such as carbohydrates, to the plant), which will provide more nitrogen and enable them to better resist this perturbation.

Soil Microbial Diversity Did Not Influence the Plant-Microorganism Interactions

Initial soil diversity had respectively little and no effect on root bacterial and fungal alpha-diversities. Only rhizosphere bacterial and fungal diversities were impacted by soil microbial diversity, but not by herbivory. When evaluating abiotic and biotic stresses (Kissoudis et al., 2014), some studies established a positive relationship between an ecosystem stability-resilience and its diversity, while others found opposite relationships or none at all (Griffiths and Philippot, 2013). In our study, the resilience of microbial communities did not seem to come from their initial diversity: microbial results were globally similar at high and low initial diversities and plant chemistry was not impacted by these levels of initial diversity either, as found by Lachaise et al. (2017). We hypothesize that our “low” microbial diversity condition might still have

been too rich to properly assess the effect of soil diversity and that a stronger dilution may be necessary in future studies.

CONCLUSION

In summary, herbivory led to root chemical changes, involving carbohydrates and sulfur-containing compounds, which partly shaped belowground microbial communities and particularly the phyla of γ -*Proteobacteria* and *Firmicutes* and a couple of their affiliated genera. It indicates that a plant suffering from herbivory emits either defensive and/or nutritive compounds that influence the recruitment of soil microorganisms by the rhizosphere and the roots.

Our results encourage the determination of the precise identity and functions of microorganisms responding to herbivory, using more accurate primers and metatranscriptomic approaches, and the understanding of the feedback-loop existing between these identified microorganisms and the plant chemistry modified by herbivory. This future research could represent the next step toward the transition from correlation to causation in order to develop sustainable and innovative plant protection strategies.

AUTHOR CONTRIBUTIONS

MO, CM, and A-MC conceived and designed research. LL and A-YG-E prepared the soil used in the experiment. MO, CP, and VC conducted plant harvest. JL and CM conducted respectively the molecular and bioinformatics analyses. MO performed metabolomics analysis, advised by NM. MO did the statistical analyses and interpreted data, advised by LL, MH, AO, and DP. MO wrote the manuscript, which was commented and approved by all authors.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fevo.2018.00091/full#supplementary-material>

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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7. Supplemental materials

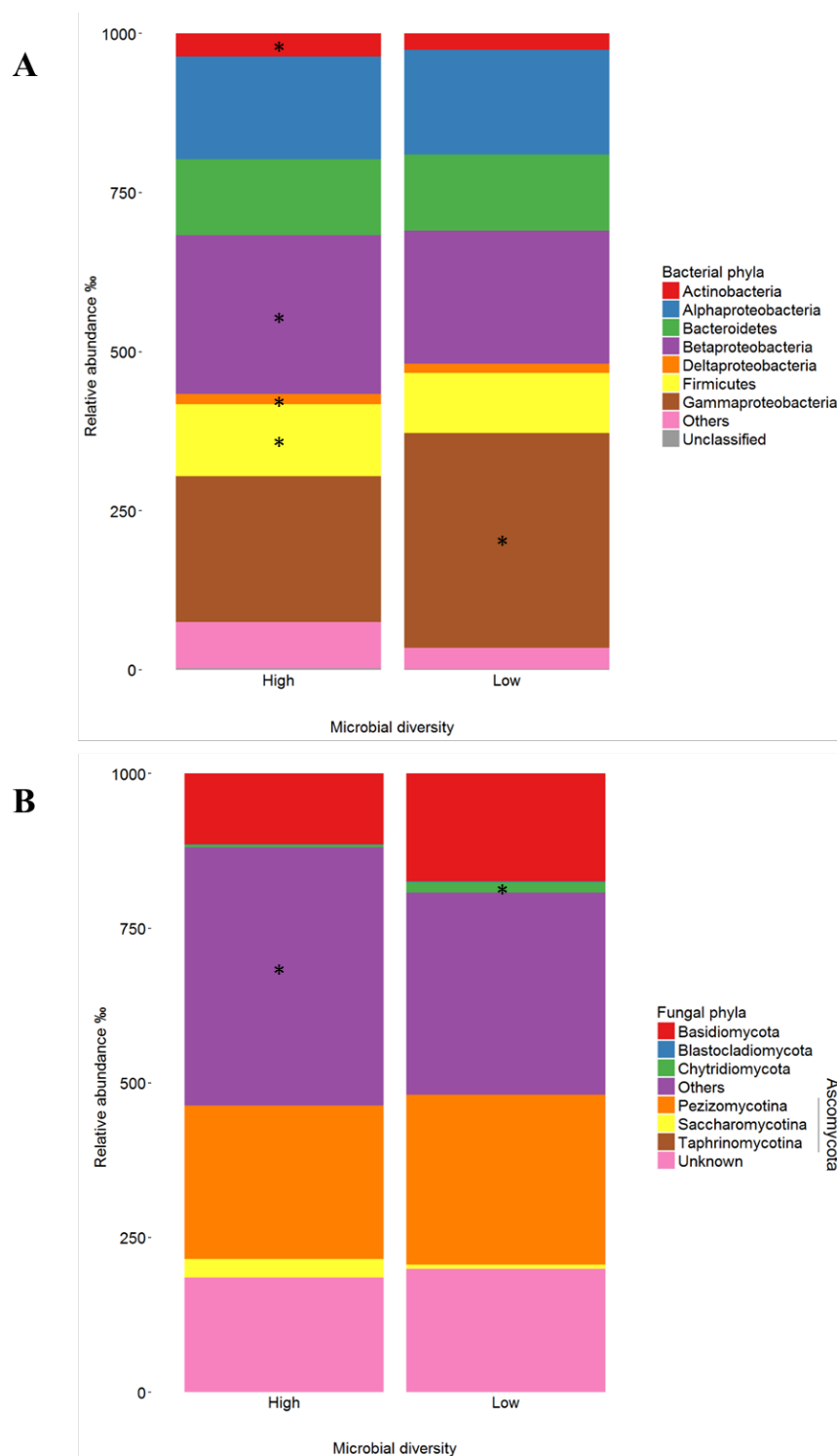


Figure S1 Mean relative abundance of dominant bacterial (A) and fungal (B) phyla from the bulk soil compartment of high and low microbial diversities before sowing. Black asterisks indicate significantly higher abundances in one modality compared to the other one.

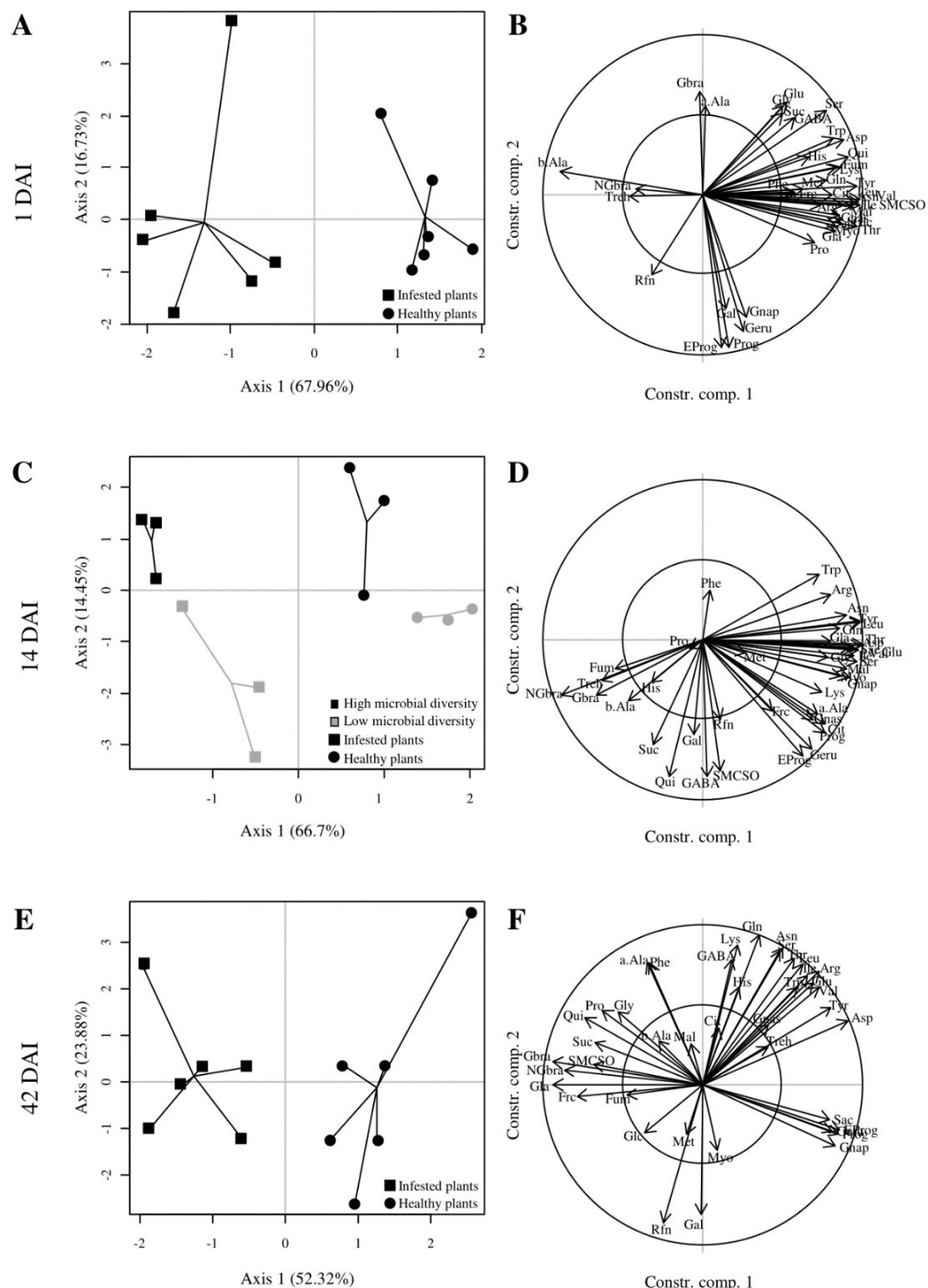


Figure S2 Redundancy Discriminant Analyses (RDA) of root metabolomic composition. Score plots (A, C, E) and correlation circles (B, D, F) of the metabolites are given for 1 (A, B), 14 (C, D) and 42 (E, F) days after infestation (“DAI”). Healthy and infested plants are represented respectively by squares and circles. When shown, high and low soil microbial diversities are colored in black and grey.

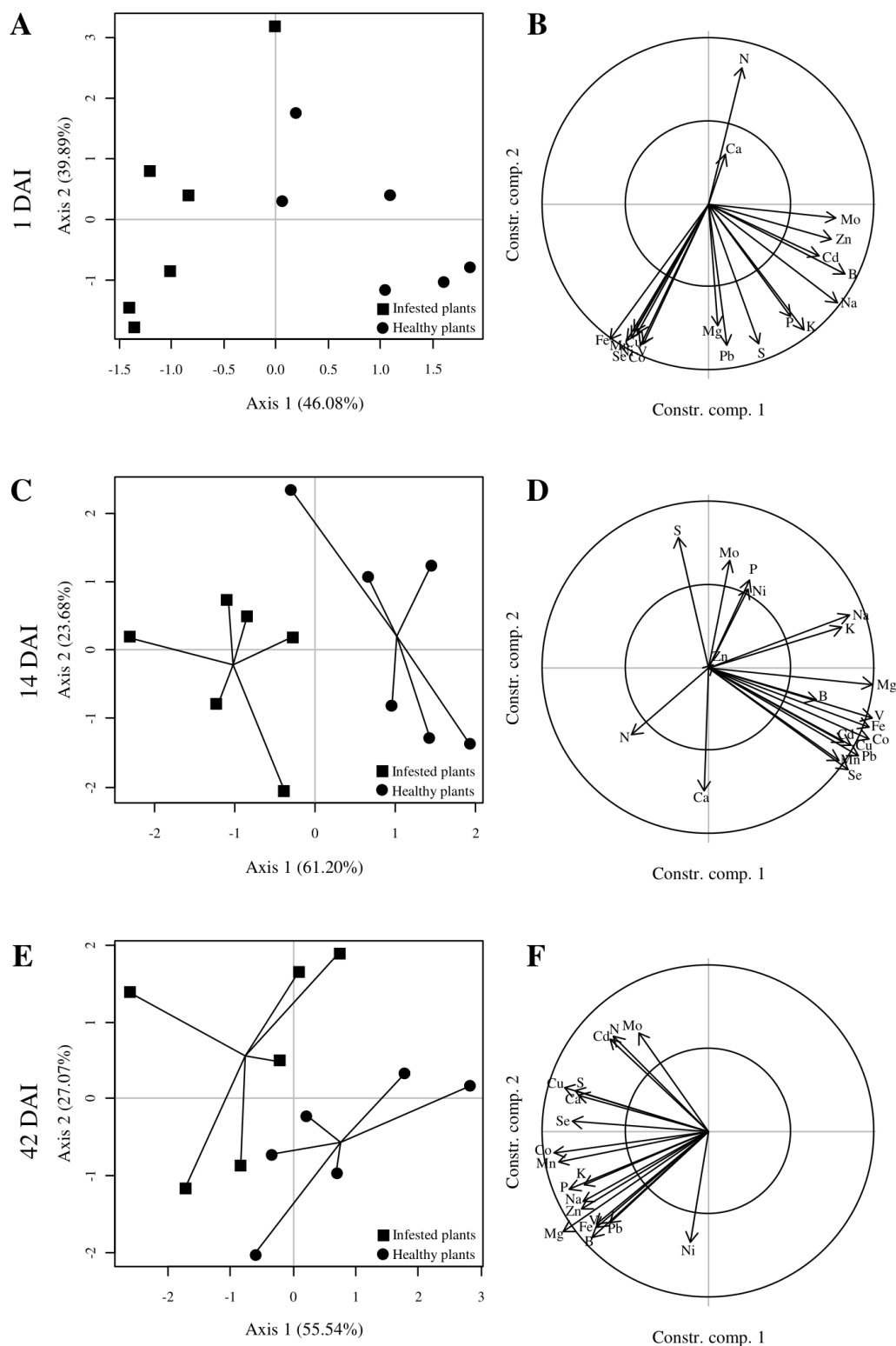


Figure S3 Graphs of the Redundancy Discriminant Analyses (RDA) of root elemental composition. Score plots (A, C, E) and correlation circles (B, D, F) of the chemical elements are given for 1 (A, B), 14 (C, D) and 42 (E, F) days after infestation (“DAI”). Healthy and infested plants are represented respectively by squares and circles. An absent barycenter means no difference between the represented profiles.

Table S1 Alpha-diversity indices in initial bulk soil. Mean value (\pm se) of richness (i.e. number of OTUs), Shannon (i.e. diversity) and Pielou (i.e. evenness) indices for bacteria and fungi at both high and low initial soil diversities are displayed in the table. Outputs of one-way anova realized on the indices are also shown: F value, degree of freedom (df) and P value (in bold when significant).

Microbial diversity		High	Low	Test results
Bacteria	Richness	341.3 \pm 2.51	304.8 \pm 4.87	F = 44.09; df = 1; P < 0.001
	Shannon index	3.82 \pm 0.02	3.55 \pm 0.03	F = 34.92; df = 1; P < 0.001
	Pielou index	0.65 \pm 0.0051	0.62 \pm 0.0069	F = 15; df = 1; P < 0.010
Fungi	Richness	114 \pm 2.69	86 \pm 3.56	F = 39.26; df = 1; P < 0.001
	Shannon index	2.37 \pm 0.01	1.96 \pm 0.12	F = 9.97; df = 1; P < 0.010
	Pielou index	0.50 \pm 0.003	0.44 \pm 0.02	F = 4.76; df = 1; P < 0.05

Table S2 Root bacterial genera significantly influenced by the plant treatment. The table below gives the mean absolute abundance (\pm se) of root bacterial genera associated to healthy and infested plants of either high or low initial soil microbial diversity, as well as the output (P value in bold when significant) of the genewise negative binomial generalized linear models performed on these genera. Displayed genera are significantly impacted ($P < 0.05$) by the plant treatment, at either 1, 14 or 42 days after infestation ("DAI"), and at either high or low diversity.

Time	Phyla	Genera	High microbial diversity		Low microbial diversity	
			Healthy	Infested	Healthy	Infested
			P value			P value
1						
14	<i>Acidobacteria</i>	<i>Candidatus Solibacter</i>	4.33 \pm 1.67	1.00 \pm 0.58	4.50 \pm 2.50	0 \pm 0
		<i>Streptomyces</i>	256.00 \pm 54.31	180.00 \pm 54.28	110.50 \pm 18.50	138.67 \pm 4.98
	<i>α-proteobacteria</i>	<i>Agrobacterium</i>	1.33 \pm 0.88	188.33 \pm 59.25	0.50 \pm 0.50	1.33 \pm 1.33
		<i>Brevundimonas</i>	33.00 \pm 4.36	95.00 \pm 38.97	2.50 \pm 1.50	13.67 \pm 13.67
		<i>Kaistia</i>	2.00 \pm 1.00	5.67 \pm 1.76	0.50 \pm 0.50	2.33 \pm 0.67
		<i>Mesorhizobium</i>	84.00 \pm 12.74	42.67 \pm 7.67	106.50 \pm 40.50	25.00 \pm 12.5
		<i>Ochrobactrum</i>	3.00 \pm 1.53	16.67 \pm 7.13	0 \pm 0	0 \pm 0
		<i>Rhizobium</i>	230.00 \pm 42.57	177.33 \pm 5.70	135.00 \pm 12.00	166.33 \pm 53.62
		<i>Rhodoplanes</i>	47.00 \pm 5.86	6.67 \pm 1.86	44.00 \pm 18.00	7.67 \pm 1.20
	<i>Bacteroidetes</i>	<i>Chryseobacterium</i>	0 \pm 0	124.67 \pm 124.67	0 \pm 0	105.33 \pm 87.49
		<i>Dyadobacter</i>	58.67 \pm 11.26	95.33 \pm 8.19	67.50 \pm 6.50	57.33 \pm 25.46
		<i>Hydroitalea</i>	4.00 \pm 0.58	2.00 \pm 1.00	0 \pm 0	2.33 \pm 1.45
		<i>Terrimonas</i>	20.33 \pm 2.03	5.33 \pm 3.93	25.00 \pm 4.00	2.33 \pm 0.88
	<i>β-proteobacteria</i>	<i>Comamonas</i>	0 \pm 0	56.00 \pm 41.36	0.50 \pm 0.50	0 \pm 0
		<i>Protonivibrio</i>	1.33 \pm 0.88	0 \pm 0	4.00 \pm 0	0 \pm 0
	<i>Chlorobi</i>	<i>Prosthecochloris</i>	0.33 \pm 0.33	0.33 \pm 0.33	10.00 \pm 0	0.67 \pm 0.67
	<i>δ-proteobacteria</i>	<i>Archangium</i>	5.33 \pm 2.60	18.33 \pm 5.55	0 \pm 0	0 \pm 0
		<i>Cystobacter</i>	1.00 \pm 0.58	1.00 \pm 0.58	5.50 \pm 3.50	0 \pm 0
	<i>Firmicutes</i>	<i>Anaerosporeobacter</i>	0 \pm 0	3.67 \pm 1.76	0 \pm 0	0 \pm 0
		<i>Blautia</i>	0 \pm 0	3.67 \pm 0.33	1.00 \pm 1.00	0 \pm 0
		<i>Clostridialisibacter</i>	1.00 \pm 0.58	0.33 \pm 0.33	3.00 \pm 2.00	0 \pm 0
		<i>Clostridium</i>	18.00 \pm 2.52	913.67 \pm 203.8	7.50 \pm 1.50	5.33 \pm 0.67
		<i>Exiguobacterium</i>	117.67 \pm 20.22	20.00 \pm 7.09	59.50 \pm 3.50	24.67 \pm 2.60
		<i>Faecalibacterium</i>	6.67 \pm 1.86	0 \pm 0	4.50 \pm 1.50	0 \pm 0
		<i>Lachnospira</i>	0 \pm 0	8.33 \pm 3.84	0 \pm 0	0 \pm 0
		<i>Paenibacillus</i>	236.67 \pm 47.57	743.67 \pm 48.14	149.50 \pm 38.50	2370.67 \pm 440.88
		<i>Planomicrobium</i>	8.67 \pm 6.17	3.00 \pm 1.00	1.0 \pm 1.0	2.67 \pm 0.88
		<i>Pseudobutyrvibrio</i>	0 \pm 0	5.00 \pm 5.00	0.50 \pm 0.50	0 \pm 0
		<i>Roseburia</i>	0 \pm 0	3.33 \pm 1.33	0 \pm 0	0 \pm 0
		<i>Solibacillus</i>	0.67 \pm 0.33	1.00 \pm 0.58	2.50 \pm 1.50	0 \pm 0
		<i>Streptococcus</i>	3.00 \pm 1.00	2.33 \pm 0.33	0.50 \pm 0.50	3.00 \pm 1.00

Table S2 (continued)

Time	Phyla	Genera	High microbial diversity			Low microbial diversity		
			Healthy	Infested	P value	Healthy	Infested	P value
14	<i>Firmicutes</i>	<i>Thermoflavimicrobium</i>	2.00 ± 1.00	0 ± 0	0.237	0 ± 0	1.33 ± 1.33	0.016
		<i>Tumebacillus</i>	99.00 ± 63.79	23.67 ± 11.55	0.716	4.00 ± 0	0 ± 0	0.018
	<i>γ-proteobacteria</i>	<i>Aeromonas</i>	0 ± 0	88.67 ± 72.72	< 0.001	0 ± 0	0.33 ± 0.33	0.217
		<i>Enterobacter</i>	4.00 ± 0.58	15.33 ± 3.28	< 0.001	2.00 ± 0	6.67 ± 0.33	0.001
		<i>Plesiomonas</i>	0 ± 0	13.33 ± 13.33	0.042	0 ± 0	0.33 ± 0.33	0.296
		<i>Pseudomonas</i>	357.67 ± 35.18	3361.67 ± 342.19	< 0.001	162.00 ± 4.00	2939.67 ± 869.07	< 0.001
		<i>Serratia</i>	1.00 ± 0.58	6.00 ± 3.46	0.071	1.00 ± 0	7.67 ± 5.78	0.023
		<i>Stenotrophomonas</i>	12.33 ± 1.67	471.67 ± 43.66	< 0.001	10.50 ± 1.50	503.33 ± 236.45	< 0.001
		<i>Thermomonas</i>	3.67 ± 0.67	1.33 ± 0.33	0.958	2.0 ± 0	4.67 ± 1.20	0.009
		<i>Gemmatimonas</i>	77.00 ± 4.00	13.00 ± 4.04	0.038	37.50 ± 12.50	11.00 ± 2.31	0.211
	<i>Acidobacteria</i>	<i>Arthrobacter</i>	20.00 ± 5.57	3.67 ± 0.67	0.010	7.67 ± 1.76	10.00 ± 3.06	0.688
		<i>Brevibacterium</i>	115.33 ± 36.83	27.67 ± 1.76	0.005	172.67 ± 36.08	97.00 ± 16.01	0.367
		<i>Candidatus Solibacter</i>	18.67 ± 1.67	1.67 ± 1.20	0.010	4.67 ± 2.03	1.00 ± 0.58	0.198
		<i>Gaiella</i>	39.33 ± 10.17	5.00 ± 1.53	< 0.001	11.67 ± 3.38	7.00 ± 1.53	0.567
		<i>Holophaga</i>	15.00 ± 1.53	4.00 ± 1.53	0.107	20.00 ± 0	4.00 ± 1.00	0.010
		<i>Planosporangium</i>	10.00 ± 2.08	1.33 ± 0.33	0.017	3.67 ± 1.45	2.00 ± 1.00	0.652
		<i>Aminobacter</i>	3.67 ± 0.67	16.67 ± 7.84	0.001	11.33 ± 0.33	10.67 ± 4.81	1
		<i>Asticcacaulis</i>	12.67 ± 5.04	78.33 ± 6.36	< 0.001	9.33 ± 2.85	46.00 ± 13.75	0.002
		<i>Bradyrhizobium</i>	144.00 ± 24.02	41.33 ± 6.67	0.007	105.00 ± 18.25	43.33 ± 3.28	0.032
		<i>Brevundimonas</i>	10.00 ± 2.52	211.00 ± 40.28	< 0.001	0 ± 0	46.67 ± 13.04	< 0.001
42	<i>α-proteobacteria</i>	<i>Caulobacter</i>	24.67 ± 8.45	36.33 ± 8.35	0.264	24.00 ± 8.50	88.67 ± 12.25	0.046
		<i>Dongia</i>	42.00 ± 3.06	8.33 ± 2.60	0.003	4.00 ± 2.65	3.33 ± 0.88	0.981
		<i>Kaistia</i>	1.67 ± 1.20	78.67 ± 3.18	< 0.001	0.67 ± 0.67	122.00 ± 44.23	< 0.001
		<i>Labrys</i>	1.00 ± 0.58	13.67 ± 3.18	0.001	4.33 ± 2.40	39.67 ± 25.21	0.004
		<i>Ochrobactrum</i>	3.33 ± 0.88	50.33 ± 8.09	< 0.001	0 ± 0	1.00 ± 0.58	0.197
		<i>Paracoccus</i>	0.33 ± 0.33	0.33 ± 0.33	0.905	0 ± 0	19.67 ± 17.67	0.002
		<i>Pseudolabrys</i>	70.00 ± 8.50	16.00 ± 1.00	0.003	33.33 ± 6.64	14.00 ± 1.73	0.094
		<i>Rhizobium</i>	166.33 ± 3.53	249.33 ± 38.88	0.046	128.00 ± 10.54	226.67 ± 67.47	0.125
		<i>Sinorhizobium</i>	1.67 ± 0.88	13.67 ± 4.06	0.013	0.67 ± 0.33	1.00 ± 0.58	0.813
		<i>Spllingopyxis</i>	35.00 ± 10.82	137.67 ± 30.75	< 0.001	28.33 ± 3.38	128.67 ± 42.25	0.002
	<i>Bacteroidetes</i>	<i>Chitinophaga</i>	64.00 ± 11.06	174.00 ± 46.23	0.002	54.00 ± 2.89	166.67 ± 82.49	0.012
		<i>Chryseobacterium</i>	0 ± 0	45.67 ± 43.19	0.013	0 ± 0	10.67 ± 10.67	0.094
		<i>Flavobacterium</i>	78.33 ± 21.61	304.33 ± 163.82	0.002	76.33 ± 28.01	121.67 ± 36.20	0.392
		<i>Flexibacter</i>	316.00 ± 49.24	93.67 ± 10.40	0.007	151.00 ± 24.06	56.00 ± 14.57	0.007
		<i>Pedobacter</i>	64.33 ± 18.48	171.00 ± 8.62	0.012	36.00 ± 14.36	179.67 ± 112.79	0.004

Table S2 (continued)

Table S2 (Continued)

Time	Phyla	Genera	High microbial diversity			Low microbial diversity		
			Healthy	Infested	P value	Healthy	Infested	P value
42	<i>β-proteobacteria</i>	<i>Comamonas</i>	0.33 ± 0.33	20.00 ± 8.02	0.002	0 ± 0	0 ± 0	1
		<i>Massilia</i>	1.00 ± 0	6.67 ± 2.33	0.009	0.67 ± 0.33	5.33 ± 1.86	0.049
		<i>Variovorax</i>	72.67 ± 28.81	21.33 ± 1.45	0.189	98.00 ± 31.32	23.00 ± 8.72	0.028
<i>δ-proteobacteria</i>	<i>Archangium</i>	1.67 ± 0.88	67.33 ± 30.28	<0.001	0 ± 0	0 ± 0	1	
	<i>Bacteriovorax</i>	6.67 ± 1.76	1.33 ± 1.33	0.193	0 ± 0	3.00 ± 1.53	0.019	
	<i>Desulfomicrobium</i>	3.33 ± 0.88	6.33 ± 1.76	0.200	0 ± 0	2.33 ± 0.88	0.035	
	<i>Haliangium</i>	146.33 ± 29.08	43.67 ± 8.51	0.012	316.67 ± 66.56	108.67 ± 7.36	0.005	
	<i>Stigmatella</i>	0.67 ± 0.67	32.00 ± 11.15	<0.001	0 ± 0	0 ± 0	1	
<i>Firmicutes</i>	<i>Anaerosporobacter</i>	0.67 ± 0.67	44.67 ± 12	<0.001	0 ± 0	0 ± 0	1	
	<i>Bacillus</i>	3566.33 ± 861.89	955.67 ± 30.87	0.003	4081.33 ± 513.79	2350.67 ± 377.84	0.334	
	<i>Blautia</i>	0.67 ± 0.67	32.33 ± 15.10	0.009	2.33 ± 2.33	12.00 ± 7.57	0.352	
	<i>Clostridium</i>	15.67 ± 2.03	2323.33 ± 419.26	<0.001	13.67 ± 4.91	22.00 ± 8.19	0.333	
	<i>Exiguobacterium</i>	80.33 ± 10.59	51.33 ± 11.14	0.887	62.00 ± 13.11	126.00 ± 7.55	0.028	
	<i>Geobacillus</i>	12.00 ± 3.21	1.67 ± 0.33	0.017	9.33 ± 6.44	1.33 ± 0.67	0.028	
	<i>Lachnospira</i>	0 ± 0	86.33 ± 36.24	<0.001	0.33 ± 0.33	0 ± 0	0.536	
	<i>Paenibacillus</i>	188.33 ± 54.28	452.00 ± 81.21	0.017	200.67 ± 58.17	1756.67 ± 356.79	<0.001	
	<i>Pseudobutyrvibrio</i>	0 ± 0	15.33 ± 7.51	0.007	0 ± 0	1.00 ± 1.00	0.304	
	<i>Roseburia</i>	0.67 ± 0.67	9.67 ± 2.6	0.003	0 ± 0	1.67 ± 1.20	0.102	
	<i>Ruminococcus</i>	0 ± 0	15.00 ± 7.81	0.002	0 ± 0	0 ± 0	1	
	<i>Sarcina</i>	0.33 ± 0.33	7.33 ± 2.85	0.003	0.33 ± 0.33	0.67 ± 0.33	0.759	
	<i>Solibacillus</i>	2.00 ± 1.15	20.33 ± 10.4	0.001	0.67 ± 0.33	1.00 ± 0.58	0.786	
	<i>Sporosarcina</i>	157.67 ± 32.12	33.67 ± 1.45	0.003	401.00 ± 54.92	194.00 ± 36.17	0.201	
	<i>Streptococcus</i>	1.33 ± 0.88	3.67 ± 1.20	0.146	1.33 ± 1.33	8.67 ± 4.10	0.043	
<i>Turicibacter</i>	0 ± 0	11.33 ± 7.54	0.003	0.33 ± 0.33	0.33 ± 0.33	0.986		
<i>γ-proteobacteria</i>	<i>Aeromonas</i>	0.67 ± 0.33	11.00 ± 4.62	0.011	0.67 ± 0.33	0 ± 0	0.346	
	<i>Dyella</i>	19.00 ± 4.04	72.67 ± 48.70	0.010	27.33 ± 12.84	37.33 ± 11.35	0.749	
	<i>Enterobacter</i>	2.00 ± 0	6.00 ± 2.00	0.028	2.00 ± 0.58	6.33 ± 1.45	0.102	
	<i>Pseudomonas</i>	187.67 ± 22.10	849.00 ± 95.70	<0.001	128.00 ± 14.36	819.67 ± 128.22	<0.001	
	<i>Stenotrophomonas</i>	5.00 ± 1.53	301.00 ± 65.16	<0.001	9.33 ± 3.71	229.33 ± 112.12	<0.001	
	<i>Steroidobacter</i>	10.00 ± 1.53	1.33 ± 0.67	0.017	7.00 ± 1.15	3.00 ± 1.00	0.345	
	<i>Thermomonas</i>	2.00 ± 1.00	5.67 ± 2.33	0.044	1.00 ± 0	9.00 ± 2.89	0.002	
<i>Gemmatimonadetes</i>	<i>Gemmatimonas</i>	49.67 ± 5.81	7.33 ± 2.03	<0.001	42.33 ± 10.37	16.67 ± 3.76	0.052	

Table S3 Rhizosphere bacterial genera significantly influenced by the plant treatment. The table below gives the mean absolute abundance (\pm se) of rhizosphere bacterial genera associated to healthy and infested plants of either high or low initial soil microbial diversity, as well as the output (P value in bold when significant) of the genewise negative binomial generalized linear models performed on these genera. Displayed genera are significantly impacted ($P < 0.05$) by the plant treatment, at either 1, 14 or 42 days after infestation ("DAI"), and at either high or low diversity.

Time	Phyla	Genera	High microbial diversity		Low microbial diversity		P value
			Healthy	Infested	Healthy	Infested	
1							
14	<i>Actinobacteria</i>	<i>Solirubrobacter</i>	14.00 ± 1.73	3.67 ± 1.20	9.67 ± 0.33	3.33 ± 0.67	0.335
		<i>Streptomyces</i>	49.67 ± 2.03	86.67 ± 7.31	27.33 ± 5.36	43.67 ± 9.26	0.016
	<i>α-proteobacteria</i>	<i>Agrobacterium</i>	0 ± 0	58.00 ± 32.62	0 ± 0	0 ± 0	1
		<i>Devosia</i>	233.33 ± 19.75	87.67 ± 12.73	252.33 ± 7.42	111.00 ± 14.19	0.029
		<i>Hyphomicrobium</i>	46.33 ± 2.96	29.33 ± 1.76	98.33 ± 16.71	34.33 ± 1.33	0.047
		<i>Ochrobactrum</i>	1.00 ± 0.58	8.00 ± 1.53	0.33 ± 0.33	0.33 ± 0.33	0.939
		<i>Rhodobium</i>	48.33 ± 1.86	16.33 ± 1.86	35.33 ± 5.24	16.00 ± 2.65	0.324
	<i>Bacteroidetes</i>	<i>Rhodoplanes</i>	134.00 ± 1.00	71.33 ± 3.18	135.33 ± 15.84	51.67 ± 6.01	0.011
		<i>Arcicella</i>	0.67 ± 0.67	3.00 ± 1.00	1.00 ± 0.58	9.00 ± 3.51	0.038
		<i>Chryseobacterium</i>	0 ± 0	38.67 ± 38.67	0 ± 0	59.33 ± 56.85	0.015
		<i>Flavisolibacter</i>	97.33 ± 5.17	34.00 ± 5.51	224.00 ± 63.37	101.33 ± 22.56	0.324
		<i>Flexibacter</i>	39.67 ± 6.57	219.67 ± 35.34	49.33 ± 9.21	77.00 ± 18.5	0.162
	<i>β-proteobacteria</i>	<i>Fluviicola</i>	2.67 ± 0.33	11.67 ± 1.76	6.00 ± 2.52	40.00 ± 33.00	0.029
		<i>Pontibacter</i>	6.33 ± 1.45	0.67 ± 0.67	12.33 ± 0.33	4.33 ± 0.67	0.419
<i>Polaromonas</i>		3.67 ± 0.33	5.33 ± 1.76	0 ± 0	2.00 ± 0.58	0.034	
<i>Variovorax</i>		23.00 ± 1.15	37.00 ± 7.64	15.00 ± 1.15	24.33 ± 3.71	0.039	
<i>Chlamydiae</i>		0 ± 0	0.67 ± 0.67	1.00 ± 0.58	17.33 ± 10.35	0.011	
	<i>δ-proteobacteria</i>	<i>Rhaddochlamydia</i>					
		<i>Bacteriovorax</i>	20.00 ± 8.39	11.33 ± 2.03	2.00 ± 1.15	13.00 ± 1.53	0.011
	<i>Firmicutes</i>	<i>Bacillus</i>	3803.67 ± 120.27	3428.67 ± 258.59	3115.33 ± 230.12	3469.00 ± 32.33	0.016
		<i>Clostridium</i>	45.00 ± 5.20	396.00 ± 112.34	29.67 ± 3.48	17.00 ± 3.06	0.704
		<i>Lachnospira</i>	0 ± 0	4.33 ± 2.96	0 ± 0	0 ± 0	1
		<i>Paenibacillus</i>	305.33 ± 8.69	365.00 ± 17.21	210.67 ± 41.25	699.33 ± 127.18	<0.001
		<i>Planomicrobium</i>	12.33 ± 1.67	25.33 ± 4.91	9.00 ± 1.73	19.67 ± 2.96	0.014
	<i>γ-proteobacteria</i>	<i>Staphylococcus</i>	1.00 ± 0	6.67 ± 2.03	0.67 ± 0.67	0 ± 0	0.476
		<i>Turicibacter</i>	3.00 ± 0.58	17.00 ± 6.08	0 ± 0	0.67 ± 0.67	0.331
		<i>Acinetobacter</i>	0.33 ± 0.33	5.33 ± 0.33	1.00 ± 0.58	2.33 ± 1.33	0.383
		<i>Aeromonas</i>	3.00 ± 1.15	32.33 ± 16.76	0.33 ± 0.33	6.33 ± 4.37	0.023
		<i>Enterobacter</i>	2.33 ± 1.20	15.33 ± 4.98	0.67 ± 0.33	6.00 ± 2.08	0.015
		<i>Pseudomonas</i>	257.33 ± 25.77	1245 ± 204.75	547.00 ± 93.93	1145.67 ± 345.56	0.013

Table S3 (continued)

Time	Phyla	Genera	High microbial diversity		Low microbial diversity	
			Healthy	Infested	Healthy	Infested
						P value
14	<i>γ-proteobacteria</i>	<i>Silanimonas</i>	3.00 ± 0.58	0 ± 0	1.67 ± 0.33	2.00 ± 0
		<i>Stenotrophomonas</i>	35.00 ± 4.62	233.33 ± 90.47	48.67 ± 11.17	150.67 ± 56.53
42	<i>Actinobacteria</i>	<i>Gaiella</i>	111.67 ± 23.34	28.33 ± 9.35	49.00 ± 8.96	30.00 ± 6.93
		<i>Afpia</i>	75.33 ± 10.09	31.67 ± 6.17	110.00 ± 10.5	45.67 ± 7.75
		<i>Agrobacterium</i>	0.33 ± 0.33	13.67 ± 7.67	0 ± 0	1.00 ± 1.00
		<i>Asticcacaulis</i>	6.33 ± 0.67	23.33 ± 5.81	5.00 ± 0.58	8.33 ± 3.38
		<i>Brevundimonas</i>	10.00 ± 2.00	77.67 ± 22.40	3.00 ± 1.15	13.33 ± 3.38
	<i>α-proteobacteria</i>	<i>Devosia</i>	222.33 ± 15.62	112.33 ± 16.91	200.33 ± 21.71	92.67 ± 7.62
		<i>Kaistia</i>	2.67 ± 2.19	39.67 ± 11.92	4.00 ± 0.58	38.67 ± 10.73
		<i>Mesorhizobium</i>	161.00 ± 11.85	75.00 ± 11.15	158.00 ± 35.03	66.00 ± 8.02
		<i>Ochrobactrum</i>	2.33 ± 0.33	16.00 ± 7.21	0.33 ± 0.33	0 ± 0
		<i>Skermanella</i>	0 ± 0	4.33 ± 1.20	1.00 ± 0.58	0.67 ± 0.67
	<i>Bacteroidetes</i>	<i>Bacteroides</i>	0 ± 0	2.67 ± 1.20	0.67 ± 0.33	0.33 ± 0.33
		<i>Chitinophaga</i>	62.67 ± 9.77	111.67 ± 39.25	90.67 ± 18.19	98.00 ± 16.44
		<i>Flexibacter</i>	53.67 ± 2.33	199.67 ± 16.80	46.33 ± 18.22	89.00 ± 28.16
		<i>Fluviicola</i>	1.33 ± 0.67	19.00 ± 4.16	2.67 ± 2.19	11.67 ± 7.75
	<i>β-proteobacteria</i>	<i>Variovorax</i>	35.00 ± 4.73	44.33 ± 0.88	17.67 ± 2.19	35.33 ± 2.96
	<i>δ-proteobacteria</i>	<i>Bacteriovorax</i>	15.33 ± 1.33	12.00 ± 5.77	1.00 ± 0.58	7.00 ± 2.52
		<i>Nannocystis</i>	2.00 ± 1.15	7.67 ± 0.88	1.00 ± 1.00	1.33 ± 0.67
		<i>Stigmatella</i>	0.33 ± 0.33	5.00 ± 3.61	0 ± 0	0 ± 0
	<i>Firmicutes</i>	<i>Anaerosporeobacter</i>	1.00 ± 0	11.67 ± 6.17	1.33 ± 0.88	0 ± 0
		<i>Bacillus</i>	3671.67 ± 96.15	3851.00 ± 216.77	3198.33 ± 141.02	3861.33 ± 187.79
		<i>Clostridium</i>	35.00 ± 5.13	825.67 ± 153.88	25.33 ± 0.88	28.33 ± 3.93
		<i>Lachnospira</i>	0 ± 0	13.67 ± 7.67	0.33 ± 0.33	0.67 ± 0.33
		<i>Paenibacillus</i>	301.67 ± 15.56	347.00 ± 21.73	229.67 ± 27.96	730.33 ± 43.98
		<i>Staphylococcus</i>	0.33 ± 0.33	11.00 ± 2.08	1.00 ± 0.58	1.00 ± 0.58
		<i>Turicibacter</i>	2.33 ± 0.88	29.33 ± 2.85	2.00 ± 0.58	1.00 ± 0.58
	<i>γ-proteobacteria</i>	<i>Cellvibrio</i>	0.67 ± 0.33	5.00 ± 0.58	3.67 ± 1.76	1.67 ± 0.33
		<i>Dokdonella</i>	26.33 ± 5.04	92.33 ± 18.21	30.33 ± 17.13	59.67 ± 47.32
		<i>Stenotrophomonas</i>	20.00 ± 1.53	76.00 ± 36.86	39.67 ± 11.05	50.00 ± 15.72
	<i>Verrucomicrobia</i>	<i>Prosthecobacter</i>	0 ± 0	1.67 ± 0.88	0.33 ± 0.33	2.67 ± 0.33
						0.090

Table S5 Output of variance analyses on linear models of root metabolites. The table below presents the outcome of variance analyses (F test): F value, degree of freedom (df) and P value (in bold when significant) are given for treatment (healthy vs infested plants) and soil microbial diversity (high vs low diversity) factors, as well as the interaction between both factors, at each sampling time (1, 14 and 42 days after infestation “DAI”).

Group	Metabolites and symbols	Time	Treatment			Soil microbial diversity			Treatment:Soil microbial diversity		
			F	df	P	F	df	P	F	df	P
AA Acid	Aspartate Asp	1 DAI	21.665	1	0.010	0.003	1	0.997	1.680	1	0.855
		14 DAI	93.734	1	<0.001	10.432	1	0.092	0	1	0.997
		42 DAI	14.651	1	0.038	1.176	1	0.706	0.644	1	0.828
	γ -aminobutyric acid GABA	1 DAI	1.228	1	0.383	0.369	1	0.932	1.452	1	0.855
		14 DAI	2.062	1	0.241	9.527	1	0.092	2.877	1	0.971
		42 DAI	0	1	0.996	0.001	1	0.980	1.026	1	0.828
	Glutamate Glu	1 DAI	1.549	1	0.331	0.633	1	0.932	0.758	1	0.855
		14 DAI	46.317	1	<0.001	5.721	1	0.157	0.015	1	0.971
		42 DAI	2.960	1	0.248	0.385	1	0.831	0.647	1	0.828
Aliphatic	Alpha Alanine a.Ala	1 DAI	0.352	1	0.636	0.458	1	0.932	1.337	1	0.855
		14 DAI	9.127	1	0.042	11.857	1	0.092	3.472	1	0.971
		42 DAI	2.139	1	0.340	4.474	1	0.541	1.610	1	0.828
	Beta Alanine b.Ala	1 DAI	97.900	1	<0.001	2.121	1	0.932	0.008	1	0.958
		14 DAI	3.581	1	0.140	0.014	1	0.951	0.144	1	0.971
		42 DAI	1.323	1	0.457	1.199	1	0.706	2.907	1	0.828
	Glycine Gly	1 DAI	3.262	1	0.164	0.015	1	0.997	4.091	1	0.855
		14 DAI	14.529	1	0.019	0.134	1	0.848	3.587	1	0.971
		42 DAI	7.206	1	0.108	0.216	1	0.831	0.519	1	0.828
	Isoleucine Ile	1 DAI	28.244	1	0.010	0	1	0.997	1.241	1	0.855
		14 DAI	25.964	1	0.006	3.751	1	0.202	0.220	1	0.971
		42 DAI	3.451	1	0.226	0.251	1	0.831	0.526	1	0.828
	Leucine Leu	1 DAI	45.006	1	0.008	0.494	1	0.932	0.487	1	0.855
		14 DAI	89.789	1	<0.001	4.228	1	0.181	0.027	1	0.971
		42 DAI	3.089	1	0.246	0.002	1	0.980	0.793	1	0.828
	Proline Pro	1 DAI	6.493	1	0.074	0.795	1	0.932	0.066	1	0.940
		14 DAI	0.304	1	0.631	0.055	1	0.885	0.037	1	0.971
		42 DAI	6.057	1	0.129	2.022	1	0.661	2.285	1	0.828
	Valine Val	1 DAI	38.932	1	0.008	0.542	1	0.932	1.779	1	0.855
		14 DAI	30.135	1	0.006	4.490	1	0.181	0.093	1	0.971
		42 DAI	3.819	1	0.206	0.667	1	0.754	0.559	1	0.828

Table S5 (continued)

Group	Metabolites and symbols	Time	Treatment			Soil microbial diversity			Treatment: Soil microbial diversity		
			F	df	P	F	df	P	F	df	P
AA Amidic	Asparagine Asn	1 DAI	23.443	1	0.010	0.11	1	0.957	0.398	1	0.855
		14 DAI	49.152	1	<0.001	2.333	1	0.297	4.343	1	0.971
		42 DAI	0.724	1	0.543	0.828	1	0.754	0.670	1	0.828
	Glutamine Gln	1 DAI	10.381	1	0.042	1.426	1	0.932	0.355	1	0.855
		14 DAI	23.735	1	0.008	3.374	1	0.203	1.362	1	0.971
		42 DAI	0.223	1	0.784	0.058	1	0.973	0.877	1	0.828
Aromatic	Phenylalanine Phe	1 DAI	1.081	1	0.406	0.626	1	0.932	4.708	1	0.855
		14 DAI	0.053	1	0.845	0.289	1	0.799	1.954	1	0.971
		42 DAI	5.237	1	0.153	0.037	1	0.973	0.061	1	0.904
	Tryptophane Trp	1 DAI	11.122	1	0.040	4.137	1	0.932	0.038	1	0.958
		14 DAI	18.171	1	0.012	0.185	1	0.818	1.489	1	0.971
		42 DAI	0.957	1	0.511	0.217	1	0.831	0.013	1	0.959
	Tyrosine Tyr	1 DAI	74.786	1	<0.001	0.434	1	0.932	0.010	1	0.958
		14 DAI	140.942	1	<0.001	3.439	1	0.203	0.010	1	0.971
		42 DAI	3.917	1	0.206	0.655	1	0.754	0.095	1	0.904
Basic	Arginine Arg	1 DAI	7.382	1	0.064	0.277	1	0.932	0.506	1	0.855
		14 DAI	20.139	1	0.010	2.156	1	0.310	2.686	1	0.971
		42 DAI	3.864	1	0.206	0.667	1	0.754	0.486	1	0.828
	Histidine His	1 DAI	5.745	1	0.084	1.190	1	0.932	4.737	1	0.855
		14 DAI	5.780	1	0.086	0.670	1	0.643	1.720	1	0.971
		42 DAI	0.010	1	0.970	1.561	1	0.677	0.002	1	0.976
	Lysine Lys	1 DAI	6.055	1	0.079	0.016	1	0.997	2.628	1	0.855
		14 DAI	3.373	1	0.148	3.425	1	0.203	0.015	1	0.971
		42 DAI	0.031	1	0.932	0.293	1	0.831	0.155	1	0.904
Hydroxylic	Serine Ser	1 DAI	7.157	1	0.065	0.152	1	0.932	1.739	1	0.855
		14 DAI	60.618	1	<0.001	10.885	1	0.092	0.525	1	0.971
		42 DAI	0.607	1	0.576	0.196	1	0.831	0.118	1	0.904
	Threonine Thr	1 DAI	21.902	1	0.010	0.171	1	0.932	0.297	1	0.855
		14 DAI	175.317	1	<0.001	18.372	1	0.070	0.044	1	0.971
		42 DAI	1.323	1	0.457	0.746	1	0.754	0.058	1	0.904
Sulfur-containing	Methionine Met	1 DAI	15.998	1	0.021	0.969	1	0.932	0.392	1	0.855
		14 DAI	0.669	1	0.492	0.091	1	0.855	0.450	1	0.971
		42 DAI	0.917	1	0.511	0.401	1	0.831	0.859	1	0.828

Table S5 (continued)

Group	Metabolites and symbols	Time	Treatment			Soil microbial diversity			Treatment: Soil microbial diversity		
			F	df	P	F	df	P	F	df	P
AA	S-methyl cysteine sulfoxide SMCSO	1 DAI	2291.762	1	0.037	0.795	1	0.932	0.795	1	0.855
		14 DAI	0.830	1	0.463	9.367	1	0.092	0.096	1	0.971
		42 DAI	15.879	1	0.037	1.260	1	0.706	0.449	1	0.828
CPO	Fructose Frc	1 DAI	3.276	1	0.164	0.847	1	0.932	0.674	1	0.855
		14 DAI	0.752	1	0.476	0.408	1	0.766	1.782	1	0.971
		42 DAI	11.318	1	0.052	3.689	1	0.541	0.001	1	0.976
	Glucose Glc	1 DAI	24.415	1	0.010	0.007	1	0.997	0.098	1	0.918
		14 DAI	38.695	1	0.004	5.579	1	0.157	0.480	1	0.971
		42 DAI	1.658	1	0.412	0.005	1	0.980	0.289	1	0.883
	Raffinose Rfn	1 DAI	3.251	1	0.164	1.244	1	0.932	0.921	1	0.855
		14 DAI	0.033	1	0.862	0.320	1	0.799	0.004	1	0.975
		42 DAI	0.779	1	0.539	1.092	1	0.706	0.071	1	0.904
	Sucrose Sucr	1 DAI	23.803	1	0.010	0.450	1	0.932	0.541	1	0.855
		14 DAI	64.173	1	<0.001	6.465	1	0.142	0.137	1	0.971
		42 DAI	14.707	1	0.038	1.857	1	0.661	2.062	1	0.828
	Trehalose Treh	1 DAI	7.338	1	0.064	0.190	1	0.932	0.455	1	0.855
		14 DAI	8.655	1	0.046	0.096	1	0.855	4.172	1	0.971
		42 DAI	1.055	1	0.511	0.007	1	0.980	1.008	1	0.828
Organic acids	Citrate Cit	1 DAI	9.305	1	0.048	0.989	1	0.932	2.732	1	0.855
		14 DAI	10.194	1	0.036	12.473	1	0.092	1.541	1	0.971
		42 DAI	0	1	0.996	0.227	1	0.831	1.523	1	0.828
	Fumarate Fum	1 DAI	18.907	1	0.016	0.183	1	0.932	0	1	0.983
		14 DAI	5.017	1	0.099	0.189	1	0.818	0.698	1	0.971
		42 DAI	6.140	1	0.129	3.231	1	0.569	2.197	1	0.828
	Glycerate Gla	1 DAI	26.368	1	0.010	0.326	1	0.932	0.015	1	0.958
		14 DAI	10.209	1	0.036	0.007	1	0.951	0.045	1	0.971
		42 DAI	44.239	1	0.007	14.727	1	0.126	4.380	1	0.828
	Malate Mal	1 DAI	49.258	1	<0.001	0.164	1	0.932	0.141	1	0.918
		14 DAI	24.062	1	0.008	5.154	1	0.158	0.840	1	0.971
		42 DAI	0.051	1	0.932	2.150	1	0.661	1.561	1	0.828
	Quinate Qui	1 DAI	14.127	1	0.025	0.081	1	0.970	0.287	1	0.855
		14 DAI	3.643	1	0.140	5.175	1	0.158	0.358	1	0.971
		42 DAI	11.202	1	0.052	0.312	1	0.831	0.019	1	0.959

Table S5 (continued)

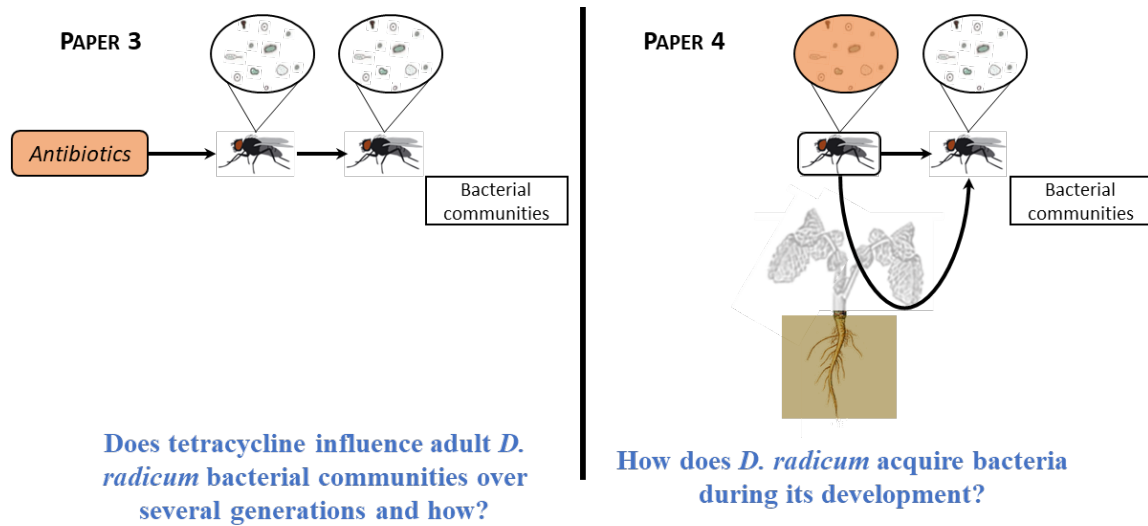
Group	Metabolites and symbols	Time	Treatment			Soil microbial diversity			Treatment: Soil microbial diversity		
			F	df	P	F	df	P	F	df	P
CPO Organic acids	Succinate Succ	1 DAI	1.879	1	0.287	0.020	1	0.997	1.854	1	0.855
		14 DAI	2.774	1	0.182	0.261	1	0.799	0.739	1	0.971
		42 DAI	8.864	1	0.081	0.036	1	0.973	1.716	1	0.828
Polyols	Galactinol Gal	1 DAI	0.014	1	0.908	0	1	0.997	2.108	1	0.855
		14 DAI	0.336	1	0.628	0.961	1	0.568	0.302	1	0.971
		42 DAI	0.909	1	0.511	0.004	1	0.980	1.700	1	0.828
	Myo Inositol Myo	1 DAI	8.356	1	0.056	2.913	1	0.932	0.608	1	0.855
		14 DAI	20.229	1	0.010	4.261	1	0.181	0.162	1	0.971
		42 DAI	0.039	1	0.932	1.733	1	0.661	0.168	1	0.904
GSL Aliphatic	Epiprogoitrin Eprog	1 DAI	0.048	1	0.854	2.534	1	0.932	0.371	1	0.855
		14 DAI	5.496	1	0.089	23.062	1	0.070	0.425	1	0.971
		42 DAI	62.267	1	<0.001	30.560	1	0.042	0.108	1	0.904
	Glucoerucin Geru	1 DAI	0.189	1	0.718	1.650	1	0.932	0.111	1	0.918
		14 DAI	3.926	1	0.138	12.332	1	0.092	0.277	1	0.971
		42 DAI	17.674	1	0.036	10.520	1	0.189	1.455	1	0.828
	Gluconapin Gnap	1 DAI	0.977	1	0.421	3.358	1	0.932	0.162	1	0.918
		14 DAI	35.991	1	0.004	7.478	1	0.126	0.296	1	0.971
		42 DAI	74.558	1	<0.001	3.915	1	0.541	1.516	1	0.828
	Progoitrin Prog	1 DAI	0.183	1	0.718	1.749	1	0.932	0.569	1	0.855
		14 DAI	14.203	1	0.019	18.759	1	0.070	0.878	1	0.971
		42 DAI	50.229	1	<0.001	25.700	1	0.042	0.440	1	0.828
Aromatic	Gluconasturtiin Gnas	1 DAI	9.534	1	0.046	0.044	1	0.997	0.007	1	0.958
		14 DAI	3.655	1	0.140	7.241	1	0.126	0.267	1	0.971
		42 DAI	0.034	1	0.932	3.960	1	0.541	0.372	1	0.846
Indolyl	Glucobrassicin Gbra	1 DAI	0.421	1	0.613	3.143	1	0.932	0.734	1	0.855
		14 DAI	6.738	1	0.069	0.765	1	0.622	0.355	1	0.971
		42 DAI	44.141	1	0.007	1.885	1	0.661	0.081	1	0.904
	Neo glucobrassicin Ngbra	1 DAI	3.778	1	0.150	1.763	1	0.932	0.137	1	0.918
		14 DAI	64.998	1	<0.001	0.004	1	0.951	0.118	1	0.971
		42 DAI	1326.362	1	<0.001	2.260	1	0.661	32.760	1	0.042

Table S6 (continued)

Group	Chemical elements and symbols	Time	Treatment			Soil microbial diversity			Treatment: Soil microbial diversity		
			F	df	P	F	df	P	F	df	P
Microelements	Manganese Mn	1 DAI	1.027	1	0.486	0.681	1	0.598	0.020	1	0.943
		14 DAI	1.590	1	0.345	0.700	1	0.579	2.498	1	0.475
		42 DAI	2.077	1	0.380	0.726	1	0.901	1.059	1	0.466
	Molybdenum Mo	1 DAI	9.189	1	0.146	6.487	1	0.167	12.873	1	0.228
		14 DAI	0.889	1	0.441	2.856	1	0.385	0.029	1	0.918
		42 DAI	9.171	1	0.144	1.021	1	0.834	0.006	1	0.942
	Nickel Ni	1 DAI	0.948	1	0.486	0.506	1	0.598	0.246	1	0.931
		14 DAI	2.431	1	0.269	1.591	1	0.439	0.949	1	0.538
		42 DAI	1.474	1	0.428	1.818	1	0.716	4.225	1	0.272
	Selenium Se	1 DAI	1.833	1	0.404	2.639	1	0.421	3.00	1	0.812
		14 DAI	3.182	1	0.216	1.897	1	0.435	0.386	1	0.661
		42 DAI	1.598	1	0.428	0.523	1	0.944	0.308	1	0.632
	Sodium Na	1 DAI	19.861	1	0.076	7.291	1	0.167	1.035	1	0.826
		14 DAI	29.044	1	0.019	4.269	1	0.304	1.625	1	0.475
		42 DAI	0	1	0.988	4.294	1	0.532	5.473	1	0.266
	Vanadium V	1 DAI	0.676	1	0.525	0.734	1	0.598	0.089	1	0.943
		14 DAI	12.801	1	0.041	0.473	1	0.579	1.638	1	0.475
		42 DAI	0.012	1	0.988	0.089	1	0.973	1.245	1	0.466
Heavy metals	Zinc Zn	1 DAI	6.851	1	0.190	0.015	1	0.906	0.051	1	0.943
		14 DAI	0.325	1	0.597	0.578	1	0.579	1.409	1	0.484
		42 DAI	0.910	1	0.532	0.072	1	0.973	1.228	1	0.466
	Cadmium Cd	1 DAI	5.880	1	0.198	0.223	1	0.730	0.257	1	0.931
		14 DAI	12.179	1	0.041	4.725	1	0.304	1.179	1	0.505
		42 DAI	4.492	1	0.185	2.771	1	0.698	2.447	1	0.401
	Lead Pb	1 DAI	0.147	1	0.723	0.579	1	0.598	0.023	1	0.943
		14 DAI	7.406	1	0.074	1.797	1	0.435	1.680	1	0.475
		42 DAI	0.059	1	0.969	0	1	0.985	0.596	1	0.524

CHAPTER IV

BACTERIAL COMMUNITIES OF A ROOT HERBIVORE



CHAPTER IV: BACTERIAL COMMUNITIES OF A ROOT HERBIVORE

Presentation of the chapter

Context

Symbiosis between insects and microorganisms have been largely studied. Studies have highlighted some of these microorganisms' beneficial effects, such as improvement of insect nutrition or increased resistance against heat or parasitoids. Usually, studying the effect of insect symbionts requires the use of antibiotics such as tetracycline, which has a broad-spectrum of action. It is known that tetracycline can be lethal for insects or have negative effect on their fitness but nothing is known about how the remaining microbial communities are affected and these communities can recover from such treatment after several generations.

The role of symbionts in the interaction of herbivorous insects with their host plants is emerging. Indeed, several studies have shown that symbionts could detoxify, reduce or suppress plant defenses, yet their origin are poorly understood. However, to date, few studies have focused on microbial transmission through vertical or horizontal transfers in herbivorous insects.

Approach

Two studies were conducted in this chapter. In a first study (paper 3), the aims were i) to determine the effect of tetracycline on the bacterial communities of the cabbage root fly (*D. radicum*) and ii) to assess the bacterial reconstruction after two generations of treatment on an untreated generation. To do so, we compared bacterial communities i) between control (i.e. "C") and antibiotic-treated flies (i.e. "A") and ii) between flies issued from four lines, that differed only by their antibiotic history (i.e. "CCC", "CAC", "ACC", "AAC"), using 16S primers and Illumina MiSeq sequencing on fly adult stage.

In a second study (paper 4), the aim was to evaluate both vertical and horizontal transmission of bacteria in *D. radicum* using a high resolution metabarcoding approach based on *gyrB* primers and Illumina MiSeq sequencing. To do so, we compared different development stages of *D. radicum*. Microbial communities of adult flies, their eggs (sterilized and unsterilized), following third instar larvae (starved and fed), empty pupae and eggs (sterilized and unsterilized) of the following generation were compared to investigate vertical

transmission. Horizontal transmission was assessed by comparing larval stages with root and rhizosphere samples.

Main results

The first study showed that tetracycline decreased bacterial diversity in flies but not to the point of turning into sterilized (i.e. aposymbiotic) insects. The application of antibiotic also induced a shift in the bacterial composition, in term of both abundance and frequency, that were still visible on the untreated generation, which parents and/or grandparents were treated. Flies with antibiotic history shared bacterial genera, potentially tetracycline-resistant and transmissible.

The second study showed that bacterial composition varied across the development stages of the cabbage root fly and that several bacterial species are assumed to be vertically transmitted from mothers to eggs (*Serratia marcescens* and *Acinetobacter guillouiae*) and potentially horizontally acquired from the plant roots and rhizosphere (*Pseudomonas* and *Lacibacter cauensis*). Interestingly, *Serratia*, *Acinetobacter* and *Pseudomonas* genera were also identified in the literature as carrying a *saxA* gene responsible for plant defense detoxification.

Limits

In the first experiment, C and A flies and their offspring were reared on rutabaga roots while the untreated generation was reared on turnip roots, and the way flies were sampled was also different (fed and alive vs starved and dead). Thus, these two observations made impossible the comparison between generations. Moreover, flies were not surface-sterilized, and the detected bacterial communities are from both external and internal habitats of *D. radicum*. Therefore, conclusions should be carefully made as the flies were most probably contaminated by their environment and it seems likely that environmental bacteria were detected on the fly external surface. Originally, this experiment was not designed to study the effect of tetracycline nor the bacterial construction but to create two lines: one with and one without *Wolbachia*, an intracellular symbiont of *D. radicum*. However, the population of cabbage root fly that was used did not harbor *Wolbachia* and this opened the opportunity to study microbial communities in a *Wolbachia* free population and document the effect of an antibiotic treatment on these communities.

In the second experiment, several *D. radicum* development stages were missing such as the 1st and 2nd instar larvae G+1, full pupae G+1 and adults G+1. Though the adults were sampled, their sequencing was not successful because too few reads were obtained and they did

not pass the different cleaning steps that were performed. Such problem was however not encountered in the first study. Moreover, insects were reared on rutabaga roots in laboratory before developing on oilseed rape in our experiment without any transition, which means that horizontal transmission could have occurred from these two hosts instead on focusing on one.

Both studies differed in their sequencing. In paper 3, DNA extracts were sent to the GenoScreen platform (Lille, France) where they underwent the different PCR and purification steps and where the library was prepared, using 16S primers which identify bacteria to the genus level. Demultiplexing, as well as barcode and primer trimming were performed by the platform. In paper 4, DNA extracts were amplified and purified and the library prepared in our laboratory (IGEPP, Le Rheu, France), using *gyrB* primers which identify bacteria to the species level. The library was then sent to the GeT-PlaGe platform (Toulouse, France) for sequencing. However, both demultiplexing and barcode and primer trimming were also done in our laboratory.

Perspectives

As tetracycline could affect bacterial communities over several generations, it would be relevant to determine whether it can also influence the fly phenotype. Moreover, the flies were still viable despite such microbial changes, which questions the microbial functions that could modify the fly phenotype or the potential functional redundancy of the remaining antibiotic-resistant bacteria.

Regarding microbial transmission, the missing stages should be included and investigated with the stages previously studied, to evaluate vertical transmission. However, to properly assess horizontal transmission, two series should be realized in parallel: one where insects develop on their usual rearing plants even during the experiment and one where they develop on another host plant during the experiment. Moreover, methods like green-fluorescent proteins (GFPs) could be used to precisely follow the path of bacterial transmission. Finally, moving toward functional approaches will give more information on the transmitted bacteria and on their role in tripartite interactions.

Publication status

The results of both studies are still in preparation. We submitted paper 3 to FEMS Microbiology Ecology under the title of “Long lasting effects of antibiotics on bacterial communities of adult flies”.

Paper 3

Long lasting effects of antibiotics on bacterial communities of adult flies

Submitted to FEMS Microbiology Ecology

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Author contributions

VL, DP and AMC conceived and designed research. VL carried out the experiment. MO did the bioinformatical and statistical analyses, advised by MH, LL and YO, and wrote the manuscript that was commented and approved by all authors.

Abstract:

Insect bacterial symbionts benefit their host and their study relies on the use of large spectrum antibiotics like tetracycline to weaken or suppress symbiotic communities. While antibiotics are known to have a negative impact on insect fitness, nothing is known about the precise effect of antibiotics on insect microbial communities and whether these effects last after several generations. We characterized the bacterial communities of the cabbage root fly *Delia radicum* at the adult stage in a *Wolbachia*-free population, evaluated the direct effect of tetracycline on these communities and assessed whether effects were still visible after two generations of successive antibiotic treatments.

Our experiment involved three generations of *D. radicum* where flies from the first and second generations either ingested tetracycline or not, while flies from the third generation were untreated but differed by the fact that their parents and grand-parents had or had not been treated themselves. Bacterial communities of adult flies were analyzed using 16S primers and sequenced with Illumina MiSeq.

We showed that tetracycline decreased bacterial diversity in flies but not to the point of obtaining sterilized (i.e. aposymbiotic) insects. The treatment also induced modifications in the abundance and relative frequencies of bacteria that were still visible on untreated offspring which parents and/or grandparents had been treated. Flies with an antibiotic history shared bacterial genera, potentially tetracycline-resistant and transmissible.

To go further, the transmission should be investigated by comparing several insect development stages and plant compartments to assess vertical and horizontal transmissions of *D. radicum* bacterial communities.

Keywords: cabbage root fly, *Delia radicum*, bacterial communities, tetracycline, antibiotic resistance.

1. Introduction

Insects can harbor a diversity of microbes that can profoundly influence their phenotypes and by extent, their ecology and evolution. To study symbiotic-mediated phenotypes, microbial-colonized hosts are usually compared to microbial-free hosts, which microorganisms were suppressed from the host body. The two commonly used methods are dechoriation and the use of antibiotics. In the first case, the chorion is removed to prevent larvae feeding on it after hatching and consuming the microorganisms left on the chorion by the mother during oviposition (Bakula, 1969; Wong *et al.*, 2011). In the second case, symbiotic bacteria are eliminated using antibiotics added to the insect food or water or microinjected in the insect body. While their use is controversial as to whether the observed effects come from the method used or the bacterial loss, the study of Heys *et al.* (2018) showed that a low dose of antibiotics in the larval diet of *Drosophila melanogaster* is more effective than dechoriation at removing bacteria forming gut communities and has reduced effects on insect physiology. Treating larvae through their diet successfully eliminated bacteria from the gut of adults but only reduced its overall adult microbiota, which comprises both internal (i.e. gut and other organs) and external microorganisms (Heys *et al.*, 2018). Lin *et al.* (2015) tested increasing doses of five antibiotics on the larval gut microbiota of *Plutella xylostella*, an aboveground chewing insect. They showed that larval growth and development were negatively affected by all antibiotics but tetracycline was the most toxic, increasing pupal malformations and mortality. Such results also raise the question of whether the observed phenotype is due to a direct and toxic effect of the antibiotic or an indirect effect from the bacterial loss.

Tetracycline is a broad-spectrum antibiotic (Dorosz, 2017), which inhibits protein synthesis and is naturally produced by bacteria from the *Streptomyces* genus (Chopra and Roberts, 2001). It has a broad spectrum as it can eliminate a wide range of gram-positive and negative bacteria, but also atypical organisms such as chlamydiae, mycoplasmas, and rickettsiae (Chopra and Roberts, 2001). In insects, tetracycline is often used to eliminate most prevalent bacterial endosymbionts like *Wolbachia* (Li *et al.*, 2014). This bacterial genus is estimated to infest up to 52% of arthropod species (Weinert *et al.*, 2015) and has various phenotypic effects on insect hosts (Werren *et al.*, 2008; Zug and Hammerstein, 2015).

The presence of *Wolbachia* is inconvenient when studying bacterial communities in general because it tends to largely dominate the community in terms of abundance (Yong *et al.*,

2017). It can represent 63% of the community in the common bed bug (Meriweather *et al.*, 2013), and over 90% of total bacteria in mosquitoes *Culex pipiens* (Muturi *et al.*, 2016). In *Drosophila*, *Wolbachia* sequences likewise represented 38% of the total sequences and rise to over 90% in several adult samples (Staubach *et al.*, 2013). While tetracycline is used to eliminate *Wolbachia* to compare infected and free lines, how this antibiotic treatment affects the bacterial community is still an open question. Depending on the effect of this antibiotic on other elements of the symbiotic consortium, differences between *Wolbachia*-infected and free lines may not be related to *Wolbachia* but rather to its impact on the microbiota. Therefore determining the effect of tetracycline on the microbiota of an insect and whether this effect lasts over insect generations is important to understand symbiotic-mediated phenotypes.

The cabbage root fly (*Delia radicum*) is a root herbivore of Brassicaceous species, which can also harbor *Wolbachia* as a facultative symbiont. *Wolbachia* prevalence varies depending on the fly population and it can go down to 0% (Lopez, 2018). Bacterial communities have been characterized in adults of two *D. radicum* populations and both populations were dominated by the *Wolbachia* genus that largely overwhelmed the other detected genera by accounting for 80% and 97% of the retrieved sequences (Bili *et al.*, 2016). So far however, the effects of an antibiotic treatment on *D. radicum* bacterial communities in the absence of *Wolbachia* and whether these effects last over several generations remain to be unraveled.

Our study aimed first at characterizing *D. radicum* bacterial communities at the adult stage in a *Wolbachia*-free population. Then, we evaluated the direct effect of tetracycline on these communities and assessed if effects lasted after one or two generations of successive antibiotic treatments and to what extent. For that purpose, we conducted an experiment over three generations of cabbage root flies: flies from the first and second generations either ingested tetracycline or not, while flies from the third generation were not in contact with the antibiotic but differed by their family history since their parents and/or grandparents had been treated or not. Bacterial communities of adult flies were identified using 16S rDNA primers and sequenced with Illumina MiSeq.

Tetracycline being a broad-spectrum antibiotic, it is expected that such treatment greatly disturbs bacterial communities and eliminates most bacteria. Moreover, when species disappear it is usually expected that others arise to recolonize the habitat. After several generations, bacterial recolonization could be due to the transmission of tetracycline-resistant bacteria, not eliminated, from the previous treated generation (i.e. vertical transmission) and/or external contamination (i.e. horizontal transmission). As several studies have shown that bacterial communities of insects differ from males to females (Simhadri *et al.*, 2017; Yong *et al.*, 2017),

it raises the question of whether antibiotics could differently shape fly bacterial communities depending on the insect sex.

2. Materials and Methods

2.1. Fly population and rearing conditions

The cabbage root fly (“*Delia radicum*”) population used in our experiment came from 300 adults emerged from pupae collected in experimental broccoli fields in 2015 near Le Rheu (48°07'16"N, 1°47'41"O, Brittany, France) which were reared in the laboratory for several generations. In the laboratory, flies were supplied with unsterilized food (ratio 1:1:1 of sugar, milk powder and yeast) and unsterilized water (cotton moistened with water) and they were reared on rutabaga roots (*Brassica napus* subsp. *rapifera*) in a climatic room (16:8 LD, 21 ± 2°C; 60% ± 10% RH) as described in Neveu Bernard-Griffiths (1998).

2.2. Experimental setup

The experimental design is detailed in figure 1.

Generation 0 (G0) – Two days after emergence from several rutabaga roots, 100 males and 100 females were placed in a control (hereafter called ‘C’) cage (Bug Dorm-4 Insect Rearing Cage, 47.5 × 47.5 × 47.5cm) and supplied with food and water, while another 100 males and 100 females were placed in an antibiotic (hereafter called ‘A’) cage with food and water containing antibiotic. The antibiotic used was tetracycline hydrochloride powder (Sigma-Aldrich, CAS number: 64-17-5), dissolved in water to a final concentration of 0.5 mg/mL (preliminary experiments showed that this concentration was the strongest one we could use without increasing the mortality of treated individuals). Treated individuals were given tetracycline continuously during their whole adult lifespan. After 15 days of treatment, a rutabaga was placed in each cage during 48h for egg-laying and after 30 days of treatment, adult flies were captured individually by aspiration, placed in 96% ethanol and stored at –20°C until further analysis.

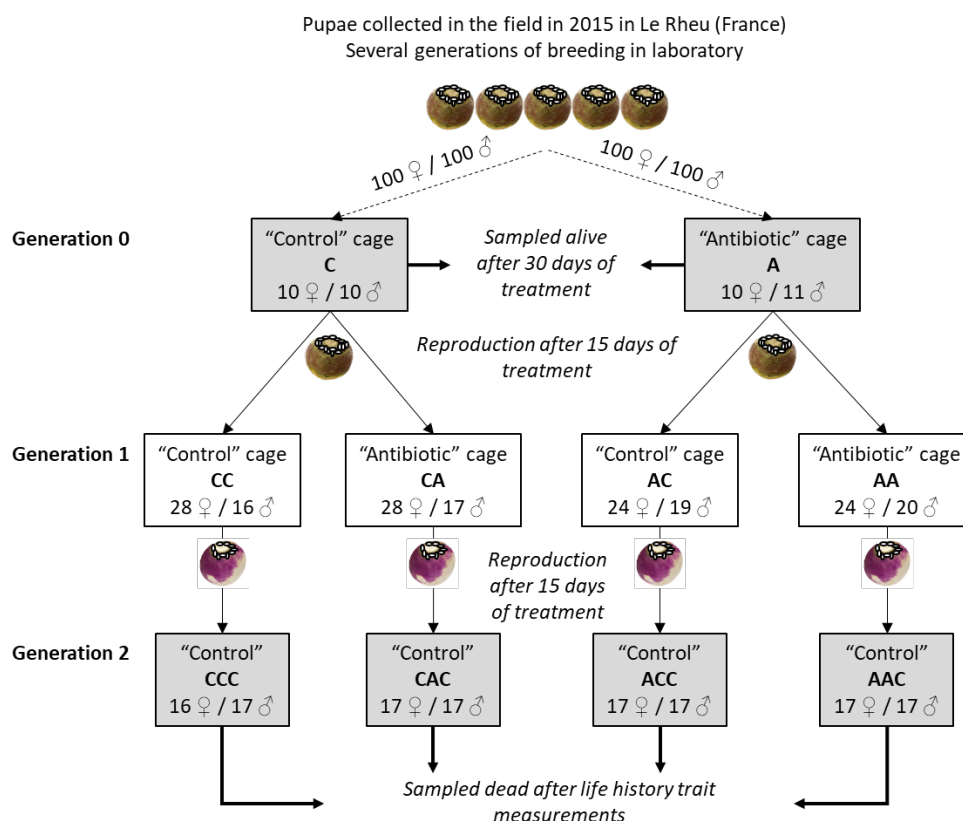


Figure 1 Experimental design used to create four lines with crossed treatments between antibiotic (A) and control (C) treatments. Grey boxes indicate the treatments and the number of samples per treatment that were sequenced for bacterial community analysis.

Generation 1 (G1) – For each G0 cage, the eggs obtained on a piece of rutabaga in each cage were placed in two different cages and developed on fresh rutabaga roots until emergence thus obtaining 4 cages. Emerging flies received either the same treatment as their parents or the alternative one (C or A) for 15 days before reproducing. They were thus exposed to four different treatments: untreated G0 and G1 (hereafter called ‘CC’ treatment’); untreated G0 but antibiotic-treated G1 (hereafter called ‘CA’ treatment’); antibiotic-treated G0 and untreated G1 (hereafter called ‘AC’ treatment’); antibiotic-treated G0 and G1 (hereafter called ‘AA’ treatment’).

For G0 and G1 flies, the food and water were changed every two days.

Generation 2 (G2) – For each of the four previous cages, 10 eggs were placed on turnip roots (*Brassica rapa* L. subsp. *rapa*, N = 40 per cage) and continued their development until adult emergence, where they all underwent the control treatment (i.e. untreated water) without food. At this stage, the four treatments were as follows: untreated G0 and G1 (hereafter called ‘CCC’ treatment’); untreated G0 but antibiotic-treated G1 (hereafter called ‘CAC’ treatment’); antibiotic-treated G0 and untreated G1 (hereafter called ‘ACC’ treatment’); antibiotic-treated

G0 and G1 (hereafter called ‘AAC’ treatment’). G2 flies were sampled once dead and stored in 96% ethanol and -20°C until further analysis.

2.3. Analyses of fly bacterial communities

2.3.1. Molecular analysis

Stored individuals were dried out on a filter paper and individually placed in a well of a semi deep 96 wells plate. DNA was extracted using 300 μL of lysis buffer: 1 M of Tris, 5 M of NaCl, 0.5 M of EDTA, 20% SDS and sterile ultrapure water; 6 μL of proteinase K (5 mg/mL) and 3 sterilized glass beads (3 mm diameter) added to each well. The plate was sealed and samples were ground during 6 min and incubated at 37°C overnight. Then, plates were rapidly centrifuged and 85 μL of NaCl (5 M) were added per well, followed by short vortexing and a 30 min centrifugation at 3,500 rpm and 4°C . Approximately 290 μL of supernatant were then transferred to a new plate containing 280 μL of 100% ice-cold ethanol and samples were homogenized by pipetting and incubated at -20°C for 1h. After a 30 min centrifugation at 3,500 rpm and 4°C , the supernatant was discarded and the pellet was washed with 200 μL of 70% ice-cold ethanol. After another centrifugation and elimination of the supernatant, samples were vacuum-dried 30 min at 30°C . Pellets of DNA were resuspended in 50 μL of sterile ultrapure water and stored at 4°C overnight. Samples were then transferred at -20°C until further analysis.

PCR amplification using the bacterial primers 799F (5'-AACMGGATTAGATACCCCKG-3') and 1223R (5'-CCATTGTAGTACGTGTGTA-3') that amplify 16S rDNA genes, library preparation and sequencing were performed at the GenoScreen platform (Lille, France) using the Illumina MiSeq platform and a 2×300 bases paired-end version. The final library at a concentration of 4 pM and the PhiX control library were loaded onto the flow cell. Sample demultiplexing and barcode suppression were performed by the GenoScreen platform using the CASAVA software (Illumina) and the PERL script “ConfigureBclToFastq.pl”, before suppressing the primers with “FLASH” tool (Magoc and Salzberg, 2011).

Raw data sets will be deposited on the European Nucleotide Archive database system.

2.3.2. Bioinformatics analysis

The dada2 workflow, based on Divisive Amplicon Denoising Algorithm (“DADA”) was used with the “dada2” R package on our samples to obtain an amplicon sequence variant

(ASV) table, which identified fine-scale variations compared to the operational taxonomic unit (OTU) table (Callahan *et al.*, 2016).

We made the following modifications to the default functions proposed by the dada2 workflow and package. After inspecting the quality profiles of reads 1 and 2 through plotting, trimming was performed at 250 reads for both read 1 and read 2 respectively where the quality score started to drop below than 30. To learn the error rates, we increased the number of samples, bases and reads taken into account by the machine-learning algorithm, with the arguments “nbases = 1e+09” and “randomize = TRUE”. Then, the dereplication, sample inference and merging steps were performed as proposed in the workflow. Lastly, the sequence lengths were inspected and only sequences which length ranged from 454 and 472 nucleotides were kept, then chimeric sequences were removed.

Taxonomic affiliations were performed using the Silva reference database, version 132.

2.3.3. Statistical analyses

Analyses were performed using the R software (R Core Team, 2018) and a 5% threshold for statistical significance.

For data manipulation, we used the “phyloseq” and “microbiome” packages (Lahti *et al.*, 2012; McMurdie and Holmes, 2013). First step, we compared A and C at G0 in order to assess the effect of tetracycline on the bacterial communities. Second step, we compared CCC, CAC, ACC and AAC in order to evaluate whether the effect of tetracycline lasted after two generations. For each test, we used the same analytical methods.

Rarefaction curves were obtained using the “ggrare” function from the “ranacapa” package (Kandlikar, 2019) to make sure that all ASVs were detected in each sample. Then samples were rarefied using the “rarefy_even_depth” function and the setting “set.seed(400)” with a sample size of 3500. Samples were normalized using per mille proportions (i.e. the sums of reads per sample transformed in 1000) instead of percentage as many ASVs had a very low abundance, and then filtered by removing ASV which proportions were lower than 1/1000.

Using rarefied, normalized and filtered data, we plotted the relative abundance of bacterial phyla and classes, obtained with the “tax_glom” function (“phyloseq” package), to visually identify the dominant phyla and classes in each treatment, using the “ggplot” function from the “ggplot2” package (Wickham, 2016).

Alpha diversity analysis was performed on normalized samples and both richness and Shannon indices were calculated using the “estimate_richness” function from the “phyloseq” package. Each index was tested against the antibiotic treatment, the sex of individuals and

interaction between these both factors using a linear model. The significance of each term in the model was determined by a F-test as a type II analysis of variance (“Anova” function, “car” package, Fox and Weisberg, 2011). When a factor was significant, the estimated marginal means (“emmeans” function, the “emmeans” package, Lenth, 2019) were calculated and a Tukey test was applied to perform pairwise comparisons using the “CLD” functions (“emmeans” package), thus to assess the differences between modalities within this factor. P values were corrected using the “False Discovery Rate” (FDR) as multiple comparisons were performed (“p.adjust” function). Plotting required the “ggplot” function.

Beta diversity or community structure analysis was performed on normalized and filtered samples. To assess whether antibiotic treatment eliminates bacteria, it was necessary for ASV proportions to be turned into a 0/1 matrix, thus presence-absence table. Then, data were transformed using the Hellinger distance as it is the method used for 0/1 matrix. A transformation-based redundancy analysis (tb-RDA) was applied on these data (“rda” function from the “vegan” package) using the antibiotic treatment, the sex of individuals and interaction between these both factors to build the model (Oksanen *et al.*, 2016). The “RVAideMemoire” package (Hervé, 2016b) was used to perform a type II permutation F-test for constrained multivariate analyses to test the significance of each term in the model (“MVA.anova” function), pairwise comparisons when a factor was significant to assess the differences between modalities within this factor (pairwise.factorfit” function) and for plotting the data (“MVA.plot” function).

By using generalized linear model (GLM) with binomial error and a logit link function, the presence of a given ASV was tested against antibiotic treatment, individual sex and interaction between both factors. A likelihood-ratio test was performed on the model to test the significance of each term in the model and then pairwise comparisons when a factor was significant to evaluate the differences between the modalities within this factor (“Anova”, “emmeans”, “CLD” functions), followed by FDR-corrected P values as multiple comparisons were performed.

In order to visualize community data at the different taxonomical level and map statistic using colors, we realized heat trees with the “heat_tree” or “heat_tree_matrix” functions from the “metacoder” package developed by Foster *et al.* (2017). Different heat trees were realized: i) on rarefied data to represent sample read depth per treatment; ii) on normalized and filtered data, the default statistical test (Wilcoxon rank-sum test, followed by FDR correction) was applied using the “compare_groups” function in order to visualize significant differences in taxa relative abundance between the treatments of interest; iii) on normalized and filtered data,

which were transformed into presence/absence data with an adapted function, hence replacing the Wilcoxon test in the “compare_groups” function, so that color 1 in the heat tree corresponds to taxa present in all replicate of treatment 1, color 2 for those in treatment 2 and color 3 for taxa present in all replicates of both treatments.

3. Results

3.1. Effects of antibiotic on the fly bacterial communities (A vs C at G0)

A total of 118,000 and 120,000 reads were detected in individuals of the control (C) and antibiotic (A) treatments respectively after the rarefying step (figure S1A). Following the different cleaning steps, most identified taxa are Proteobacteria ($\approx 970\text{-}900\%$, respectively for C and A), more precisely α -, δ - and γ -Proteobacteria (figure S1B). The second phylum represented was Bacteroidetes ($\approx 25\text{-}90\%$, for C and A), with the Bacteroidia class, while Firmicutes was the third and less abundant phylum ($\approx 5\text{-}10\%$, for C and A), with the Bacilli class.

Alpha diversity was assessed by evaluating the number of observed ASVs and calculating the Shannon index (figure 2A). For each index, the interaction between the treatment and the sex of individuals was not significant and therefore removed from the model. The number of observed ASVs was reduced by the treatment ($F=11.388$; $df=1$; $P=0.002$) but did not differ between sexes ($F=0.158$; $df=1$; $P=0.97$). Control flies (C treatment) had 47-100 ASVs (mean \pm se: 73 ± 3.5), while treated flies (A treatment) had only 5-141 ASVs (mean \pm se: 48 ± 9.9). Similarly, the Shannon index mean value was also reduced by the treatment (C: 3.12 ± 0.09 vs A: 1.96 ± 0.20 , $F=26.557$; $df=1$; $P<0.001$) but not by sex ($F=0.049$; $df=1$; $P=0.97$). Globally, the addition of tetracycline to drinking water decreased microbial diversity 1.6 fold in the host, but was also associated to increased variation between samples.

Beta diversity, or the bacterial community structure, was significantly driven by the treatment ($F=4.2401$; $df=1$; $P=0.001$) but not sex ($F=0.7684$; $df=1$; $P=0.686$, figure 2B). The interaction between the treatment and the sex of individuals was not significant and therefore removed from the model. Our model explained 11.63% of the total constrained variance, with the treatment accounting for 8.09% and sex for 1.46%. ASVs were dispatched in two groups according to their coordinates on the correlation circle: ASVs of group 1 (coordinates: axis 1 >

0) were associated to the C treatment and those of group 2 (coordinates: axis 1 < 0) to the A treatment (table S1).

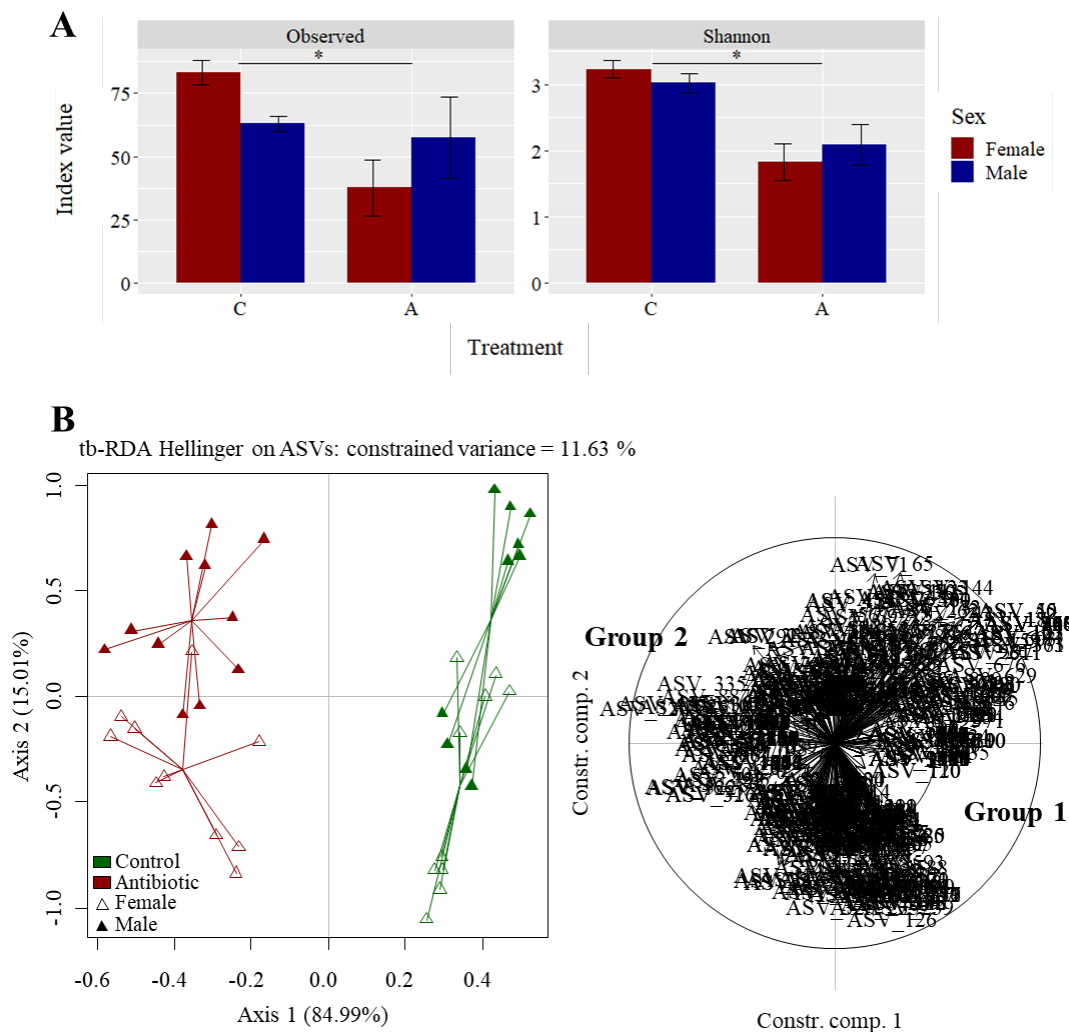


Figure 2 Bacterial diversity of G0 flies treated with tetracycline (A) or not (C). A) Alpha diversity is represented by the number of observed ASVs and the Shannon index (mean \pm se). An asterisk represents a significant difference between the two treatments. B) Community structure is represented by beta diversity analyzed using a tb-RDA performed on Hellinger-transformed presence/absence data, with sample projection on the left plot and the correlation circle of ASVs on the right one. The constrained variances explained by the axes are given in parenthesis. Group 1 aggregates ASVs which axis 1 coordinates are superior to 0 and group 2 below 0.

A total of 592 ASVs was detected and corresponded to 87 genera (table S1). *Pseudomonas* was the most assigned genus (65 ASVs), followed by *Sphingobacterium* (34), *Acinetobacter* (27) and *Flavobacterium* (22) while 81 ASVs were not assigned at the genus level, but at the family level. Among the 87 detected genera, there were 61 and 71 in the flies of treatments C and A respectively (table S2). In treated flies, 16 genera were eliminated and 26 new genera appeared compared to control flies but these genera had a very low relative

abundance, while 45 genera remained present in both treatments. When looking at the relative abundance, *Pseudomonas*, *Providencia*, *Serratia* and *Acinetobacter* were the most abundant genera in the C treatment in a decreasing order and accounted for 707‰ of the total relative abundance (table S1). Regarding the A treatment, *Serratia* was the most abundant and accounted for 546‰.

Binomial GLM was performed on the 592 detected ASVs (table S1). As sex and the interaction between the treatment and the sex of individuals were not significant in any of the analyzed ASVs, they were thus removed from the model. Among the 592 ASVs, 71 were significantly influenced by the treatment (table 1) and among the first 100 dominant ASVs, only 7 were influenced by the treatment starting at ASV 40, 55 and 56 (*Acinetobacter*), followed by ASV 76, 85, 94 (*Providencia*) and ASV 98 (*Comamonas*). Most treatment-influenced ASVs were more frequent in the C treatment and not present or very scarcely in the A treatment. Interestingly, 2 ASVs from the *Gibbsiella* genus, 2 non-assigned from the Chitinophagaceae family and another 2 non-assigned ASVs from the Enterobacteriaceae family were more frequent in the A treatment and completely absent from the C treatment. The Enterobacteriaceae (i.e. *Gibbsiella*, *Providencia*, *Rahnella*, *Serratia* and non-assigned genera) and Pseudomonadaceae (i.e. *Pseudomonas* genus) families had the most ASVs impacted by the treatment. It should be noted that the frequency of ASV in a given treatment never exceeded 55%, meaning that each ASV was present in at most about half of the replicates for a given treatment.

Table 1 Statistical outputs of ASVs with significant pairwise comparisons within the treatment, associated to the G0 flies. This table compiles taxonomic, beta diversity, frequencies and statistical information. Green and red colors correspond to ASV more frequent in the control (C) and antibiotic (A) treatments respectively, while grey shows the first 100 dominant ASVs.

Order	Family	Genus	ASV	tb-RDA group	A	C	Treatment P value
†Chitinophagales	Chitinophagaceae	NA	316	2	6/21	0/20	0.032*
			327	2	6/21	0/20	0.032*
†Flavobacteriales	Flavobacteriaceae	<i>Flavobacterium</i>	554	1	0/21	5/20	0.042*
			564	1	0/21	6/20	0.022*
			576	1	0/21	5/20	0.042*
			580	1	0/21	5/20	0.042*
†Sphingobacteriales	Sphingobacteriaceae	<i>Sphingobacterium</i>	603	1	0/21	9/20	0***
			629	1	0/21	7/20	0.016*
			832	1	0/21	6/20	0.022*
			865	1	0/21	5/20	0.042*
ªRhizobiales	Rhizobiaceae		588	1	0/21	7/20	0.016*

Order	Family	Genus	ASV	tb-RDA group	A	C	Treatment P value
		<i>Allorhizobium</i>	593	1	0/21	6/20	0.022*
		<i>Neorhizobium</i>	676	1	0/21	6/20	0.022*
		<i>Pararhizobium</i>	695	1	0/21	5/20	0.042*
^δ Bdellovibrionales	Bacteriovoracaceae	<i>Peredibacter</i>	646	1	0/21	5/20	0.042*
			726	1	0/21	6/20	0.022*
^γ Betaproteobacteriales	Burkholderiaceae	<i>Comamonas</i>	98	1	0/21	6/20	0.022*
		<i>Delftia</i>	220	1	0/21	11/20	0***
			236	1	0/21	11/20	0***
			260	1	1/21	9/20	0.022*
			276	1	0/21	9/20	0***
			597	1	0/21	6/20	0.022*
			611	1	0/21	7/20	0.016*
			725	1	0/21	6/20	0.022*
			780	1	0/21	6/20	0.022*
		<i>Gibbsiella</i>	320	2	11/21	0/20	0***
			466	2	7/21	0/20	0.016*
		NA	242	1	0/21	8/20	0***
			251	1	0/21	6/20	0.022*
			381	1	0/21	5/20	0.042*
			387	1	0/21	5/20	0.042*
			398	1	0/21	5/20	0.042*
			400	1	0/21	5/20	0.042*
			455	2	7/21	0/20	0.016*
			522	2	6/21	0/20	0.032*
		<i>Providencia</i>	76	1	0/21	11/20	0***
			85	1	0/21	11/20	0***
			94	1	0/21	11/20	0***
			108	1	0/21	11/20	0***
		<i>Rahnella</i>	119	1	0/21	9/20	0***
			123	1	1/21	11/20	0***
			131	1	0/21	9/20	0***
			141	1	0/21	10/20	0***
		<i>Serratia</i>	159	1	1/21	8/20	0.041*
			170	1	0/21	7/20	0.016*
			172	1	0/21	6/20	0.022*
			177	1	0/21	8/20	0***
			262	1	0/21	6/20	0.022*
			271	1	0/21	7/20	0.016*
^γ Pseudomonadales	Moraxellaceae	<i>Acinetobacter</i>	40	1	1/21	11/20	0***
			55	1	1/21	11/20	0***
			56	1	1/21	9/20	0.022*
			909	1	0/21	5/20	0.042*
	Pseudomonadaceae	<i>Pseudomonas</i>	105	1	1/21	11/20	0***
			122	1	1/21	11/20	0***
			134	1	0/21	11/20	0***

Order	Family	Genus	ASV	tb-RDA group	A	C	Treatment P value
γPseudomonadales	Pseudomonadaceae	<i>Pseudomonas</i>	137	1	0/21	9/20	0***
			144	1	2/21	11/20	0.016*
			147	1	0/21	11/20	0***
			152	1	0/21	9/20	0***
			158	1	0/21	11/20	0***
			176	1	2/21	11/20	0.016*
			186	1	2/21	11/20	0.016*
			239	1	1/21	9/20	0.022*
			252	1	1/21	8/20	0.041*
			363	1	0/21	10/20	0***
			371	1	0/21	10/20	0***
			394	1	0/21	7/20	0.016*
			401	1	0/21	7/20	0.016*
			405	1	0/21	5/20	0.042*
			545	1	0/21	5/20	0.042*

“.”: $0.1 < P < 0.05$; “*”: $0.05 < P < 0.01$; “***”: $0.01 < P < 0.001$; “****”: $P < 0.001$

[†]Bacteroidetes – Bacteroidia; ^αProteobacteria – alphaproteobacteria; ^δProteobacteria – deltaproteobacteria;

^γProteobacteria – gammaproteobacteria.

Using a heat tree to visualize significant variation in taxa relative abundance also revealed differences between the C and A treatments (figure 3A). Relative abundances of nine genera were significantly higher in the C treatment: *Chryseobacterium* from the Bacteroidetes; *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* from the α-Proteobacteria class; *Peredibacter* from the δ-Proteobacteria class; *Acinetobacter*, *Delftia*, *Providencia*, *Pseudomonas*, *Rahnella*, *Stenotrophomonas* from the γ-Proteobacteria class. However, the relative abundance of three genera was higher in the A than C treatment: *Gibbsiella* and *Serratia* from the Enterobacteriaceae family (class of γ-Proteobacteria) and *Fluviicola* from the Crocinitomicaceae family (phylum of Bacteroidetes).

With another heat tree, we visualized the taxa present in all flies (i.e. replicates) of one of the treatments or both (figure 3B). Interestingly, *Serratia* was the only genus to be present in all flies of both treatments. Seven genera were present in all flies of only the C treatment: the six γ-Proteobacterial genera from above (*Acinetobacter*, *Delftia*, *Providencia*, *Pseudomonas*, *Rahnella*, *Stenotrophomonas*) and also *Trabulsiella*; while no taxa was present only in all flies of the A treatment.

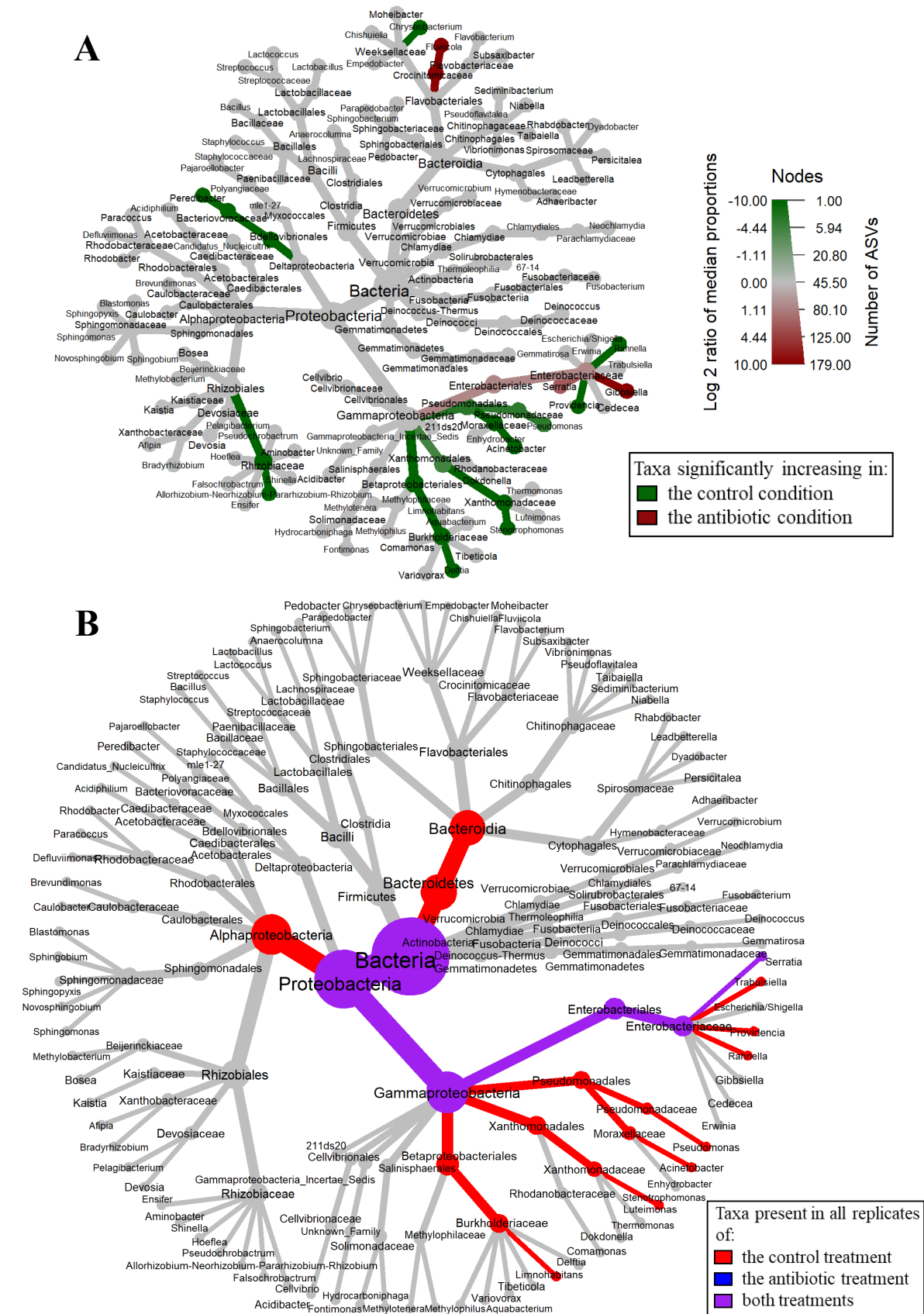


Figure 3 Heat trees comparing taxa between the antibiotic and control treatment of G0 flies. A) The color of each taxon represents the log-2 ratio of median relative abundances observed for each treatment (control or antibiotic) and only significant differences are colored. B) The color of each taxon represents the taxa presence in all flies (i.e. replicates) of a given treatment.

3.2. Bacterial community after two generations of antibiotics

After the rarefying step, we detected 116,000 reads in the CCC treatment and 119,000 reads in each of the other three treatments CAC, ACC and AAC (figure S2A). Like in G0 flies, we found mainly Proteobacteria (ranged from 813 to 931‰), Bacteroidetes (ranged from 45 to 123‰) and Firmicutes (barely detectable) in a decreasing order (figure S2B). However, only α - and γ -Proteobacteria (no δ -Proteobacteria) were detected in the dominant phylum.

Alpha diversity of the G2 flies also varied (figure 4A). For each index, the interaction between the treatment and the sex of individuals was not significant and therefore removed from the model. The number of observed ASVs was influenced by the treatment ($F=3.105$; $df=3$; $P=0.046$) but not by sex ($F=4.728$; $df=1$; $P=0.124$). Treatment AAC (mean \pm se: 38 ± 2.6) had the significantly highest number of observed ASVs while ACC (mean \pm se: 28 ± 1.7) had the lowest. The treatments CAC (mean \pm se: 33 ± 2.1) and CCC (mean \pm se: 31 ± 2.3) were similar and not different from the other two treatments (ACC and AAC). The Shannon index was impacted by the treatment as well ($F=3.083$; $df=3$; $P=0.046$), but not by sex ($F=1.239$; $df=1$; $P=0.536$). As previously, the Shannon index was higher in AAC (mean \pm se: 2.39 ± 0.09) and lower in ACC (mean \pm se: 1.98 ± 0.09) treatments. Again, CAC (mean \pm se: 2.17 ± 0.07) and CCC (mean \pm se: 2.14 ± 0.11) treatments were not statistically different and were similar to both ACC and AAC treatments.

Beta diversity was indeed driven by the treatment ($F=5.8416$; $df=3$; $P=0.001$) and very little by sex ($F=1.9125$; $df=1$; $P=0.051$, figure 4B). The interaction between the treatment and the sex of individuals was not significant and therefore removed from the model. Our model explained 12.95% of the total constrained variance, with the treatment accounting for 9.91% and sex for 1.08%. While all treatments were significantly different from one another as their community structure differed, CAC was the only treatment for which female and male profiles were separated.

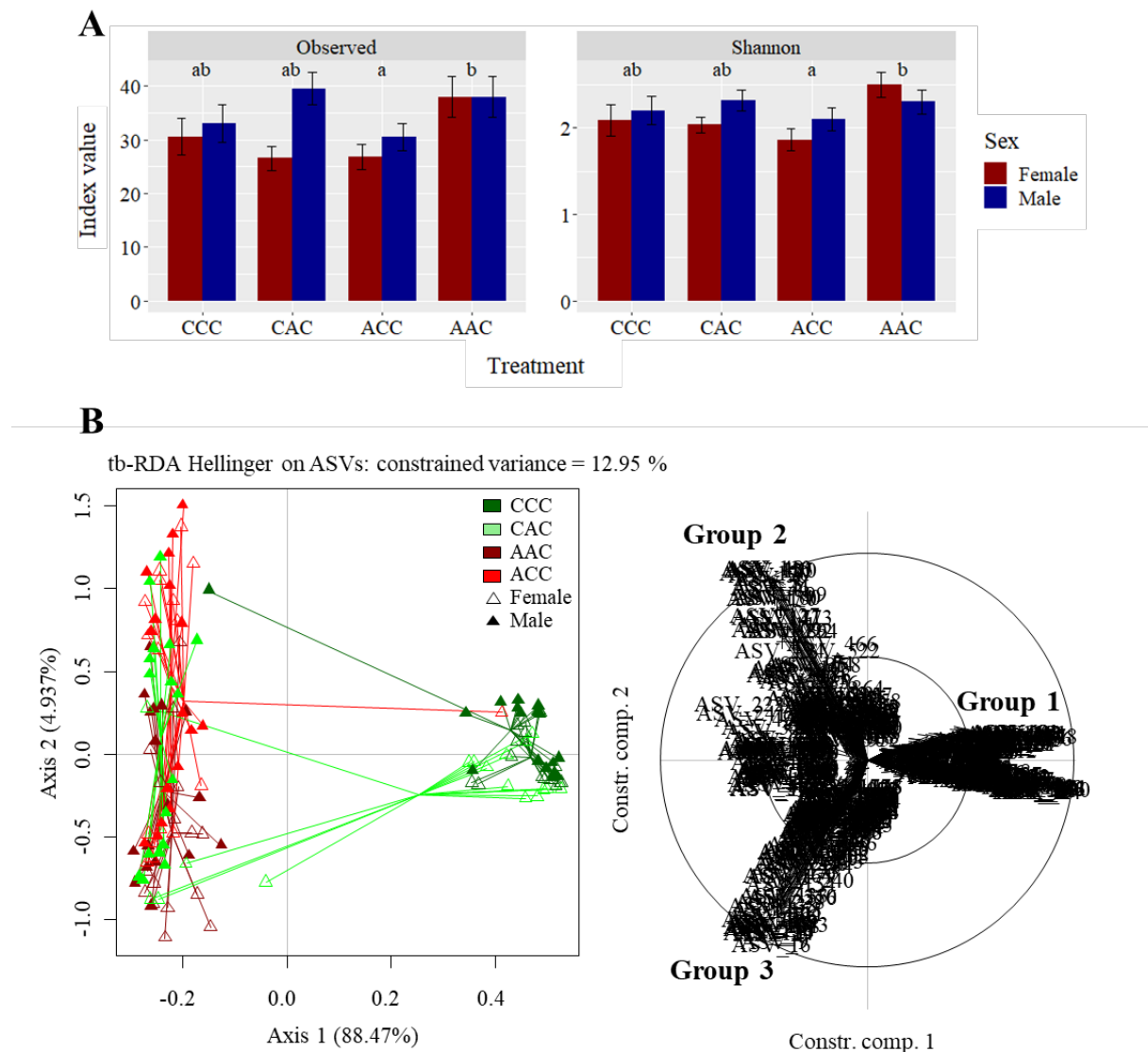


Figure 4 Bacterial diversity of G2 flies treated with tetracycline or not. A) Alpha diversity is represented by the number of observed ASVs, the Shannon, Simpson and Inverse Simpson indices (mean \pm se). Letters represent significant differences between treatments B) Community structure is represented by beta diversity analyzed using a tb-RDA performed on Hellinger-transformed presence/absence data, with sample project on the left plot and the correlation circle of ASVs on the right one. The constrained variances explained by the axes are given in parenthesis.

Moreover, three groups of ASVs could be distinguished on the tb-RDA plots according to their coordinates on the correlation circle (figure 4B, table 2). Group 1 (i.e. coordinates: axis 1 $>$ 0) aggregated ASVs associated to the CCC treatment and female CAC and a total of 76 treatment-influenced ASVs clustered in group 1 (out of 153 treatment-influenced ASVs, among the detected 489), belonging mostly to *Sphingobacterium* (Bacteroidetes phylum), *Pseudomonas* and non-assigned genera (from the γ -Proteobacteria class). Group 2 (coordinates: axis 1 $<$ 0 and axis 2 $>$ 0) clustered ASVs associated to the ACC treatment, among the 38 treatment-influenced ASVs were 11 ASVs of the *Sphingobacterium* genus and 24 ASVs from

the γ -Proteobacteria class. Last, group 3 (coordinates: axis 1 < 0 and axis 2 < 0) aggregated ASVs associated to AAC, 39 treatment-influenced ASVs, with 15 ASVs belonging to the *Sphingobacterium* genus but only 16 to the γ -Proteobacteria class. ASVs associated to male CAC seemed to stand between groups 2 and 3.

Table 2 ASV classification following tb-RDA and GLM analysis of G2 flies. Treatment-influenced ASVs were divided in 3 groups, characterized by a specific treatment: group 1, CCC and female CAC samples; group 2, ACC and male CAC samples; group 3, AAC and male CAC samples.

Phylum	Class - Genus	Number of treatment-influenced ASVs in each tb-RDA		
		Group 1	Group 2	Group 3
Bacteroidetes	Bacteroidia	20	12	16
	<i>Chryseobacterium</i>	2	1	0
	NA	0	0	1
	<i>Sphingobacterium</i>	18	11	15
Proteobacteria	Alphaproteobacteria	8	2	7
	<i>Defluviimonas</i>	0	0	1
	<i>Falsochrobactrum</i>	4	2	2
	<i>Pseudochrobactrum</i>	4	0	4
	Gammaproteobacteria	48	24	16
	<i>Acinetobacter</i>	4	3	1
	<i>Comamonas</i>	8	6	2
	<i>Erwinia</i>	1	1	0
	<i>Gibbsiella</i>	0	2	0
	NA	13	4	4
	<i>Pseudomonas</i>	15	4	5
	<i>Serratia</i>	2	1	3
	<i>Stenotrophomonas</i>	5	3	1
	Total	76	38	39

A total of 489 ASVs was detected and corresponded to 44 genera (table S3). The most assigned genus was *Pseudomonas* (177 ASVs), followed by *Sphingobacterium* (77), *Stenotrophomonas* (25) and *Falsochrobactrum* (25). This time, *Acinetobacter* and *Flavobacterium* accounted for 21 and 6 ASVs respectively. Fifty-three ASVs were however non-assigned. Among the 44 genera, 30 of them were detected in flies of the CCC treatment, 32 in the CAC, 26 in the ACC and 30 in the AAC (table S4). While 19 genera were shared between the four treatments, 6 were still exclusively present in CCC and 3 in CAC, ACC and AAC, but these 6 and 3 genera had a very low relative abundance. For the four G2 treatments, *Pseudomonas*, *Serratia*, *Sphingobacterium* and *Comamonas* were the most abundant genera in a decreasing order and they accounted for 557‰, 654‰, 657‰ and 744‰ of the total relative abundance, respectively associated with the CCC, CAC, ACC and AAC treatments (table S3).

A binomial GLM was performed on the 489 detected ASVs (table S3). As sex and the interaction between the treatment and the sex of individuals were not significant in any of the analyzed ASVs, they were removed from the model. Among the 489 ASVs, 153 were

significantly influenced by the treatment, among which 31 led to significant pairwise comparisons (table 3). Among the first 100 dominant ASVs, 18 were significantly influenced by the treatment, starting at ASV 4, and belonging to the following genera: *Sphingobacterium*, *Falsochrobactrum*, *Serratia*, *Acinetobacter*, *Pseudomonas* and *Stenotrophomonas*.

Table 3 Statistical outputs of ASVs with significant pairwise comparisons within the treatment, associated to the G2 flies. This table compiles taxonomic, beta diversity, frequencies and statistical information. Green and red colors correspond to ASV more frequent in the control (CCC) and antibiotic (AAC, ACC, CAC) treatments respectively, while grey shows the first 100 dominant ASVs. Letters correspond to statistical differences between the ASV frequency of the four treatments (AAC, ACC, CAC, CCC).

Order	Family	Genus	ASV	tb-RDA group	AAC	ACC	CAC	CCC	Treatment P value
†Sphingobacteriales	Sphingobacteriaceae	<i>Sphingobacterium</i>	91	1	0/34 ab	1/34 a	4/34 ab	12/33 b	0***
ªRhizobiales	Rhizobiaceae	<i>Falsochrobactrum</i>	88	1	0/34 ab	0/34 ab	4/34 a	14/33 b	0***
			101	1	0/34 ab	1/34 a	3/34 a	12/33 b	0***
			125	2	13/34 b	16/34 b	10/34 b	1/33 a	0***
			140	2	13/34 b	17/34 b	8/34 ab	1/33 a	0***
		<i>Pseudochrobactrum</i>	166	1	0/34 ab	1/34 a	5/34 ab	13/33 b	0***
			171	1	0/34 ab	0/34 ab	4/34 a	14/33 b	0***
‡Betaproteobacteriales	Burkholderiaceae	<i>Comamonas</i>	178	1	0/34 ab	1/34 a	5/34 a	15/33 b	0***
			198	1	0/34 ab	1/34 a	2/34 a	13/33 b	0***
			110	2	14/34 b	17/34 b	10/34 b	1/33 a	0***
			120	2	16/34 b	16/34 b	10/34 b	1/33 a	0***
			150	2	15/34 b	12/34 b	10/34 b	1/33 a	0***
			160	2	13/34 b	12/34 b	9/34 b	1/33 a	0.004**
‡Enterobacteriales	Enterobacteriaceae	NA	5	1	0/34 ab	1/34 a	6/34 ab	15/33 b	0***
			6	1	0/34 ab	0/34 ab	6/34 a	17/33 b	0***
			45	1	0/34 ab	1/34 a	6/34 ab	13/33 b	0***
			61	1	0/34 ab	1/34 a	6/34 ab	15/33 b	0***
			107	1	0/34 ab	1/34 a	2/34 a	11/33 b	0***
		<i>Serratia</i>	8	1	0/34 ab	1/34 a	6/34 ab	15/33 b	0***
			4	2	15/34 b	16/34 b	11/34 b	1/33 a	0***
‡Pseudomonadales	Moraxellaceae	<i>Acinetobacter</i>	13	1	0/34 ab	1/34 a	2/34 a	13/33 b	0***
			18	1	0/34 ab	1/34 a	3/34 a	12/33 b	0***
			48	2	16/34 b	17/34 b	9/34 b	1/33 a	0***
			56	2	13/34 b	16/34 b	9/34 b	1/33 a	0***
	Pseudomonadaceae	<i>Pseudomonas</i>	27	1	0/34 ab	1/34 a	5/34 ab	13/33 b	0***
			35	1	0/34 ab	1/34 a	5/34 ab	13/33 b	0***
			68	1	0/34 ab	0/34 ab	1/34 a	13/33 b	0***
			113	2	12/34 b	9/34 ab	7/34 ab	1/33 a	0.015*
			14	3	16/34 b	11/34 b	9/34 b	1/33 a	0***
‡Xanthomonadales	Xanthomonadaceae	<i>Stenotrophomonas</i>	74	2	13/34 b	13/34 b	10/34 b	1/33 a	0***
			77	2	10/34 b	11/34 b	10/34 b	1/33 a	0.012*

“.”: 0.1 < P < 0.05; “*”: 0.05 < P < 0.01; “***”: 0.01 < P < 0.001; “****”: P < 0.001

†Bacteroidetes – Bacteroidia; ªProteobacteria – alphaproteobacteria; ‡Proteobacteria – gammaproteobacteria.

Interestingly, 13 ASVs were significantly more frequent in the AAC, ACC and CAC treatments and barely present in the CCC treatments while 18 ASVs were more frequent in the CCC, intermediate in CAC and barely present in the AAC and ACC treatments. These two situations occurred in most genera: for the same genus, some ASVs such as *Falsochrobactrum* (from the α -Proteobacteria class) and *Comamonas*, *Serratia*, *Acinetobacter*, *Pseudomonas* (from the γ -Proteobacteria class) were more frequent in AAC, ACC and CAC while other ASVs

from the same latter genera were more frequent in the CCC treatment. We observed that frequency did not exceed 51%, meaning that none of the ASVs were present in all flies of a treatment. Before removing the sex factor from the model, we noted that ASV 99 (*Pseudomonas*) was the only one (among 489 ASVs) influenced by sex, being more frequent in males than females for all treatments.

Using differential heat trees and Wilcoxon rank-sum tests, we found no statistically significant differences in taxa relative abundance between the four treatments (figure S3).

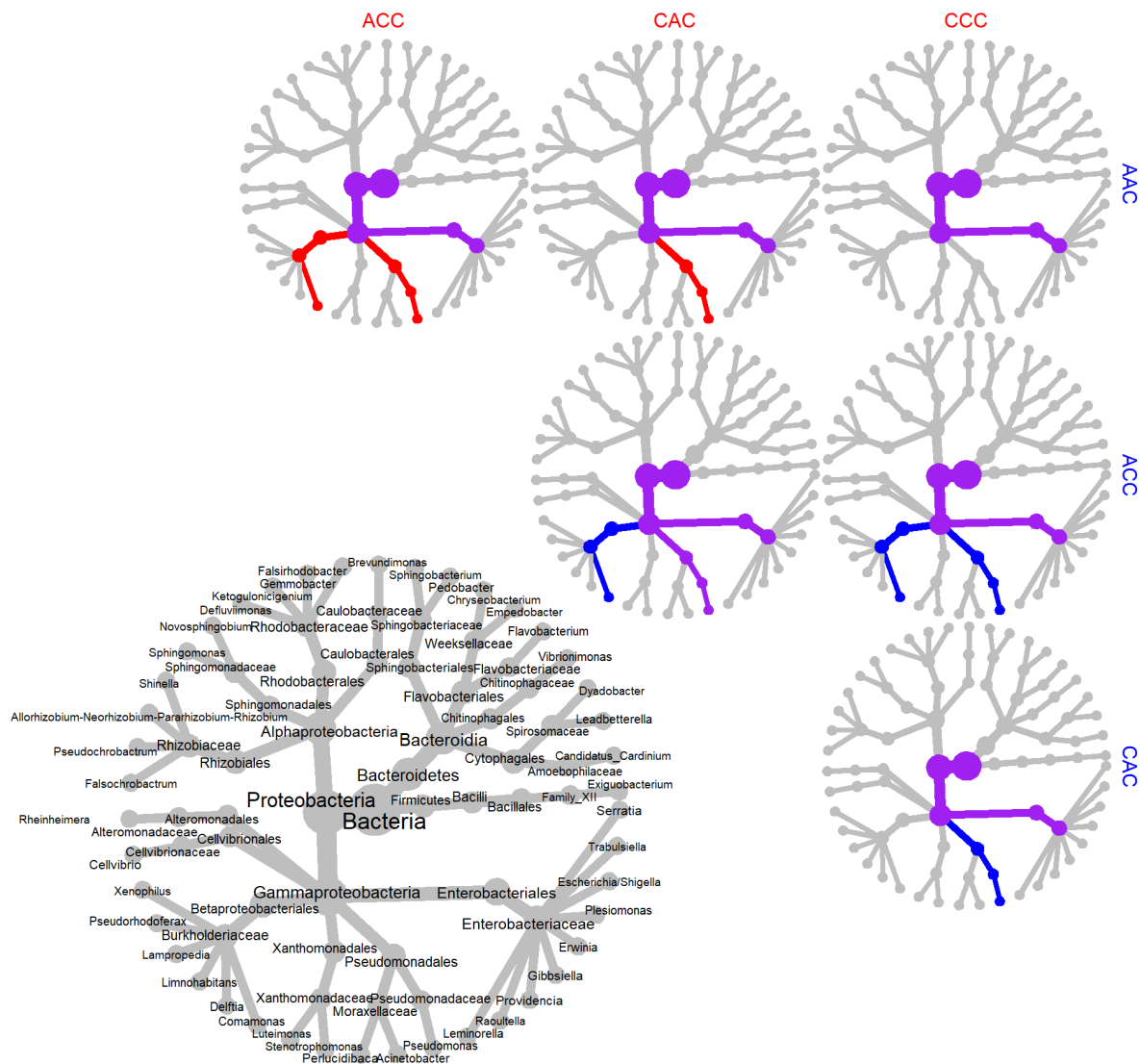


Figure 5 Heat trees comparing taxa between the antibiotic and control treatment of G2 flies. The color of each taxon represents the taxa presence in all flies (i.e. replicates) of a given treatment (AAC, ACC, CAC, CCC). Taxa colored in red are present in all flies of only the treatment shown in the column above heat trees, taxa colored in blue are present for only the treatment shown in the row on the right of heat trees and taxa colored in purple are present for both treatments. The grey tree on the lower left functions as a key for the smaller unlabeled trees.

However, when we visualized taxa presence, we noted that the family of Enterobacteriaceae was present in all flies of all treatments (figure 5). While the *Pseudomonas*

genus was present only in treatments CAC and ACC, *Comamonas* was present in all flies of ACC.

4. Discussion

Our study showed that tetracycline decreased bacterial diversity but did not suppress all bacteria. While some bacteria were eliminated, others appeared and several genera were found to be shared between control and treated flies. We also showed that effects of the antibiotic were still visible after two generations of treatment.

4.1. *Wolbachia*-free *D. radicum* show a higher microbial diversity

The *D. radicum* population used in our experiment was *Wolbachia*-free. Indeed only 4 ASVs (ASV 15, 17, 20 and 23) that could putatively corresponded to this genus were found but did not pass the cleaning steps (i.e. rarefying and filtering steps) as they were present in only 4 samples (3 G0 samples and 1 G2 sample) at a very low abundance. The bacterial communities we detected were very diverse. Bacterial communities of the G0 control treatment had a mean Shannon index of 3.12, which is twice higher than the value found in the *D. radicum* population used by Bili *et al.* (2016) where *Wolbachia* was the dominant and most abundant genus in adult flies. In this previous study, α -Proteobacteria was the most dominant phylum only because of *Wolbachia*, whereas here, in the absence of this genus, γ -Proteobacteria largely dominated the communities. Moreover, up to 88 bacterial genera were detected in the present study (table 1 and figure 3) compared to the 10 genera identified by Bili *et al.* (2016). Interestingly, our results revealed the presence of many genera that were not described so far in the studies using *Wolbachia*-infected lines and very few of the genera we detected were also found in these studies. For instance, Lukwinski *et al.* (2006) also observed the presence of *Pseudomonas*, *Serratia*, *Escherichia* and *Erwinia* but only detected 10 genera in the larval gut. Similarly, *Providencia*, *Acinetobacter*, *Pseudomonas* and *Serratia* were also observed by Welte *et al.* (2016) among 6 genera detected in the larval gut. The study of Bili *et al.* (2016) on adult flies showed the least similarities with ours as only *Bacillus* was also found in our study out of the 8 genera they identified. Still, comparisons between these studies and ours are to be made carefully as different fly populations and different protocols were used.

The reduction of *Wolbachia* also tended to enrich the bacterial communities of the parasitoid wasp *Asobara tabida* (Zouache *et al.*, 2009). However, the *Wolbachia* reduction was

achieved by a tetracycline treatment in this study, which prevented from asserting whether the bacterial community enrichment was due to the decrease of *Wolbachia* or the antibiotic application. Although not interacting directly, intracellular endosymbiont such as *Wolbachia*, could have a major impact on extracellular bacteria such as bacteria from the gut, genital organs or cuticle. Indeed, these bacteria could compete for resources with *Wolbachia* and display anti-pathogenic potential to overcome the competition (Zug and Hammerstein, 2015). *Wolbachia* was also shown recently to be able to have an influence beyond its insect host and manipulate the chemistry of the plant, where this host develops (Lopez, 2018). Our study seems to be the first one highlighting the richness of bacterial communities in a *Wolbachia*-free population of *D. radicum*. To confirm the influence of an endosymbiont like *Wolbachia* on extracellular bacterial communities it would be interesting to compare free and *Wolbachia*-infected lines obtained through breeding from the same initial population.

4.2. Tetracycline reshapes bacterial communities and does not destroy them

The daily ingestion of tetracycline in drinking water during the whole adult life of individuals decreased the diversity by 37% (i.e. Shannon index, from 3.12 to 1.96). Rosas *et al.* (2018) also observed a decrease by 2-fold of the bacterial diversity of German cockroach (*Blattella germanica*) treated with another antibiotic (i.e. rifampicin) and a shift in the composition, indicating that several bacteria were still present despite the treatment.

Tetracycline also modified the bacterial community structure and we observed that 16 genera were suppressed by the antibiotic treatment out of the 71 genera detected in treated-flies (i.e. A treatment). Similarly, a shift in bacterial composition and proportions of α - and β -Proteobacteria was observed in Hessian flies (*Mayetiola destructor*) treated with a mixture of kanamycin and streptomycin (Bansal *et al.*, 2011). The effect observed was stage dependent: the proportion of *Stenotrophomonas* decreased in treated larvae but increased in treated pupae while *Pseudomonas* proportions barely changed. Despite inducing compositional changes in bacterial community, tetracycline seemed to only have a partial effect. This partial effect we observed may be explained by the antibiotic property, as tetracycline has bacteriostatic but not bactericidal activities, hence it inhibits bacterial growth and does not kill bacteria (Jones and Morrison, 1962). As reviewed by Li *et al.* (2014) various doses of tetracycline have been applied on insects to remove their symbionts. Lin *et al.* (2015) treated *P. xylostella* larvae with a 3 mg/mL dose of tetracycline and still detected bacteria in the gut. Similarly, whitefly (*Bemisia tabaci*) treated to rifampicin still harbored traces of several symbionts (Shan *et al.*, 2016) while the symbiont *Burkholderia* was not completely removed from its southern chinch

bug (*Blissus insularis*) host after an oxytetracycline treatment of 1.4 mM or 0.6 mg/mL (Xu *et al.*, 2016). In our study, a 0.5 mg/mL dose of tetracycline was given to the flies as preliminary experiments showed that this concentration was the strongest one that could be used without increasing the mortality of treated individuals. However, as indicated by previous studies, using a stronger dose, at the expense of insect viability, may not guaranty the elimination of all bacteria.

Tetracycline induced the apparition of 26 new genera and three genera had their relative abundance increased: *Gibbsiella* and *Serratia* and *Fluviicola*. Bansal *et al.* (2011) also observed that the proportion of β -Proteobacteria, *Paenibacillus* and *Stenotrophomonas* increased in treated larvae compared to control ones, while it was the proportion of α -Proteobacteria and of *Paenibacillus* that increased in treated pupae. It could be suggested that tetracycline would hinder some very competitive bacteria, which would be taken over by newly acquired and less competitive bacteria. A first explanation to such bacteria apparition and increase after ingesting tetracycline in our experiment is that flies did not develop in a sterile environment and were not surface-sterilized as we wanted to have access to both external and internal microbial diversity. Thus, flies were most likely to have been subjected to environmental contamination while feeding or moving around during the experiment, hence potentially accumulating bacteria on their cuticle. A second explanation is that apparition of new genera and increase in abundance of the remaining bacteria following the treatment may be due to resistance to tetracycline (Chopra and Roberts, 2001). For instance, Vazirianzadeh *et al.* (2014) identified 59.4% of gram-negative bacilli and 63.4% of gram-positive cocci isolated from brown-banded cockroaches (*Supella longipalpa*) that were resistant to tetracycline among the 18 antibiotics tested. Regarding the bacteria in our study, that increased following the tetracycline treatment, *Serratia* was described as having a natural resistance to tetracycline (Dorosz, 2017) and interestingly this genus was the only one to be present in both C and A treatments. As for *Fluviivola*, the study of Wang *et al.* (2019) showed that its abundance increased in the presence of oxytetracycline while the study of Han *et al.* (2018) did not find any relationship between this genus and tetracycline resistant genes from animal manures in soil microcosms. To our knowledge, there are few records of *Gibbsiella* in insects, as this genus was mainly detected in diseased oak trees and oral cavity of bears and only *G. papilionis* was isolated from a butterfly intestinal tract (Kim *et al.*, 2013), but there is none about this genus being tetracycline resistant.

4.3. Tetracycline has long-lasting consequences on bacterial communities

Overall, effects of tetracycline were still observable after two generations of antibiotic treatment.

Bacterial communities of flies with antibiotic history tended to be similar and distinct from the ones with no antibiotic history. After two generations of treatment, *Serratia* was still more frequent in flies which parents and/or grandparents were tetracycline-treated (i.e. G2 AAC, ACC, CAC), and so were other genera like *Falsochrobactrum*, *Comamonas*, *Acinetobacter*, *Pseudomonas* and *Stenotrophomonas*. These genera could contaminate flies with an antibiotic history as they could be tolerating and/or overcoming the antibiotic (i.e. show resistance to tetracycline). According to Han *et al.* (2018), *Pseudomonas* and *Acinetobacter* seem to have significant relationships with tetracycline resistant genes while Chen *et al.* (2019) mentioned that *Comamonas* was resistant to antibiotics and even suggested that this genus could contribute to oxytetracycline biodegradation. However, studies focusing on the genus *Falsochrobactrum* are scarce. It was isolated from sheep and soil but so far, no study has shown a resistance to antibiotics (Sun *et al.*, 2019). We also noted that several ASVs of these genera (i.e. *Falsochrobactrum*, *Comamonas*, *Serratia*, *Acinetobacter*, *Pseudomonas* and *Stenotrophomonas*) were more frequent in flies with an antibiotic history, while other ASVs from these same genera were more frequent in the control flies. A potential explanation for such results is that these ASVs could correspond to different species but, as our data went down only to the genus, we lacked taxonomical precision to confirm such hypothesis. Another explanation could be that the antibiotic treatment induces mutations, which eventually lead to antibiotic resistance (Martinez, 2014). For instance, certain ASVs could have been subjected to such mutations and promoted acquisition of tetracycline resistance as previously discussed, while others have not. Simultaneously, these mutations could potentially explain the variability we observed when flies ingested tetracycline (i.e. G0 flies) by introducing, replacing or suppressing one or several nucleotides in the sequenced fragments. A single change in nucleotides leads to the identification of another ASV but ASVs that vary by one nucleotide can eventually belong to the same species.

Interestingly, the Enterobacteriaceae family was shared among the four treatments. The Enterobacteriaceae family has been largely detected in insects. For instance, γ -Proteobacteria and more precisely Enterobacteriales were the most abundant class and order in the gut of the burying beetle (Heise *et al.*, 2019), with *Serratia* being the most abundant genus. Enterobacteriaceae was the most commonly found bacterial family and one of the most

abundant in Lepidoptera (Paniagua Voirol *et al.*, 2018) as well as in mosquitoes (Muturi *et al.*, 2016), though their abundance varied depending on the environment (i.e. collection sites). In *D. radicum*, it was found that *Serratia* present in the larval gut had the ability to degrade isothiocyanates, a chemical defense emitted by the plant, so that its insect host could keep developing without being harmed (Welte *et al.*, 2016). To explain the persistence of *Serratia*, and more broadly Enterobacteriaceae in all treatments of our study, we could emit two hypotheses: i) Enterobacteriaceae bacteria were already present in control flies and they could overcome the antibiotic treatment by being tetracycline resistant and/or ii) Enterobacteriaceae bacteria are important for fly survival, and thus transmitted to the offspring.

Vertical transmission occurs when microorganisms are passed down from the parents to their offspring. Therefore, potential tetracycline-resistant bacteria could have been vertically transmitted, which might explain the similarly shared bacteria between the three G2 batches with antibiotic history. Indeed, maternal transmission can occur when the female contaminate the egg shell with its reproductive organ (Moran and Dunbar, 2006) and, upon hatching, larvae ingest bacteria from the shell (Bakula, 1969). As our experiment was not conducted under sterile conditions and our samples were not surface-sterilized prior to DNA extraction, it is highly possible for flies to have acquired bacteria through vertical transmission. For the same reasons, horizontal transmissions might also have occurred with flies acquiring bacteria from their environment and host plant. For example, the bean bug *Riptortus pedestris* acquires its symbiotic bacteria of the genus *Burkholderia* from the soil during its development (Kikuchi *et al.*, 2012) while the microbiota of the caterpillar *Mamestra brassicae* seems to be have similarities with leaf and soil microbiota (Hannula *et al.*, 2019). Pons *et al.* (2019) showed that host plant could mediate the circulation of *Serratia symbiotica* between aphids, as uninfected aphids acquired the bacteria after feeding on a plant, previously attacked by infected aphids. In our study, G0 and G1 flies were reared on rutabaga roots (*B. napus* subsp. *rapifera*) and G2 flies developed from turnip roots (*B. rapa* L. subsp. *rapa*). According to Card *et al.* (2015), there were several *Pseudomonas*, *Serratia* and *Stenotrophomonas* species that were recorded as being beneficial endophytic bacteria associated with *B. napus* roots while *Streptomyces*, and *Pseudomonas* species were associated to *B. rapa* roots. Therefore, such bacteria could be acquired from the plants.

We differentiated males from females and observed that both control and tetracycline treatments had similar effects on alpha and beta diversity, as well as ASVs of G0 male and female flies. However, male bacterial communities of the *Solanum* fruit fly were richer and

more diverse than female ones according to the study of Yong *et al.* (2017) while Simhadri *et al.* (2017) pointed out compositional differences between insect sex. In our study, we could suggest that tetracycline would have a “clean sweep” effect that would erase bacterial differences between insect sex, but the absence of difference in control flies prevent us from further hypothesizing. Still, we observed slight differences between male and female after two generations of treatments: bacterial community structure of CAC differed between insect sex and a single ASV identified as a *Pseudomonas* was influenced by the sex. Both results can be difficultly linked and using precise taxonomy at the species level may provide the missing information to explain our observations.

The long lasting modifications in fly bacterial communities induced by tetracycline could potentially have repercussions on the fly development and fitness. The study of Lopez (2018) has shown some effects and identified several life history traits affected by tetracycline one or two generations after treatment. Linking fly phenotype changes to microbial modifications is difficult but could help identifying symbionts that influence the insect phenotype.

5. Conclusion

Our study showed that tetracycline decreased bacterial diversity in flies but not to the point of turning them into sterilized (i.e. aposymbiotic) insects. The application of antibiotic also induced shifts in the bacterial composition, in terms of both abundance and frequency, and this shift were still visible on untreated offspring, which parents and/or grandparents were treated. Flies with antibiotic history shared bacterial genera, potentially tetracycline-resistant and transmissible.

As microbial transmission has not been studied in *D. radicum*, we can only hypothesize that vertical and/or horizontal transmission occurred, but it would be nice to study by comparing several insect development stages and plant compartments and by using finer taxonomic tools to identify bacteria to the species level. As the flies were viable despite these shifts, it also raises the questions of functional redundancy between the original community of untreated flies and bacteria remaining after treatment with tetracycline and of microorganism role in influencing insect life history traits.

Funding

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Acknowledgements

The authors thank Zachary Foster for his advices and help with the heat tree analysis, especially for the “presence/absence” homemade function used to plot heat trees, and Stéphane Tuffier for his help with R scripts and discussions about antibiotics.

6. Supplemental data

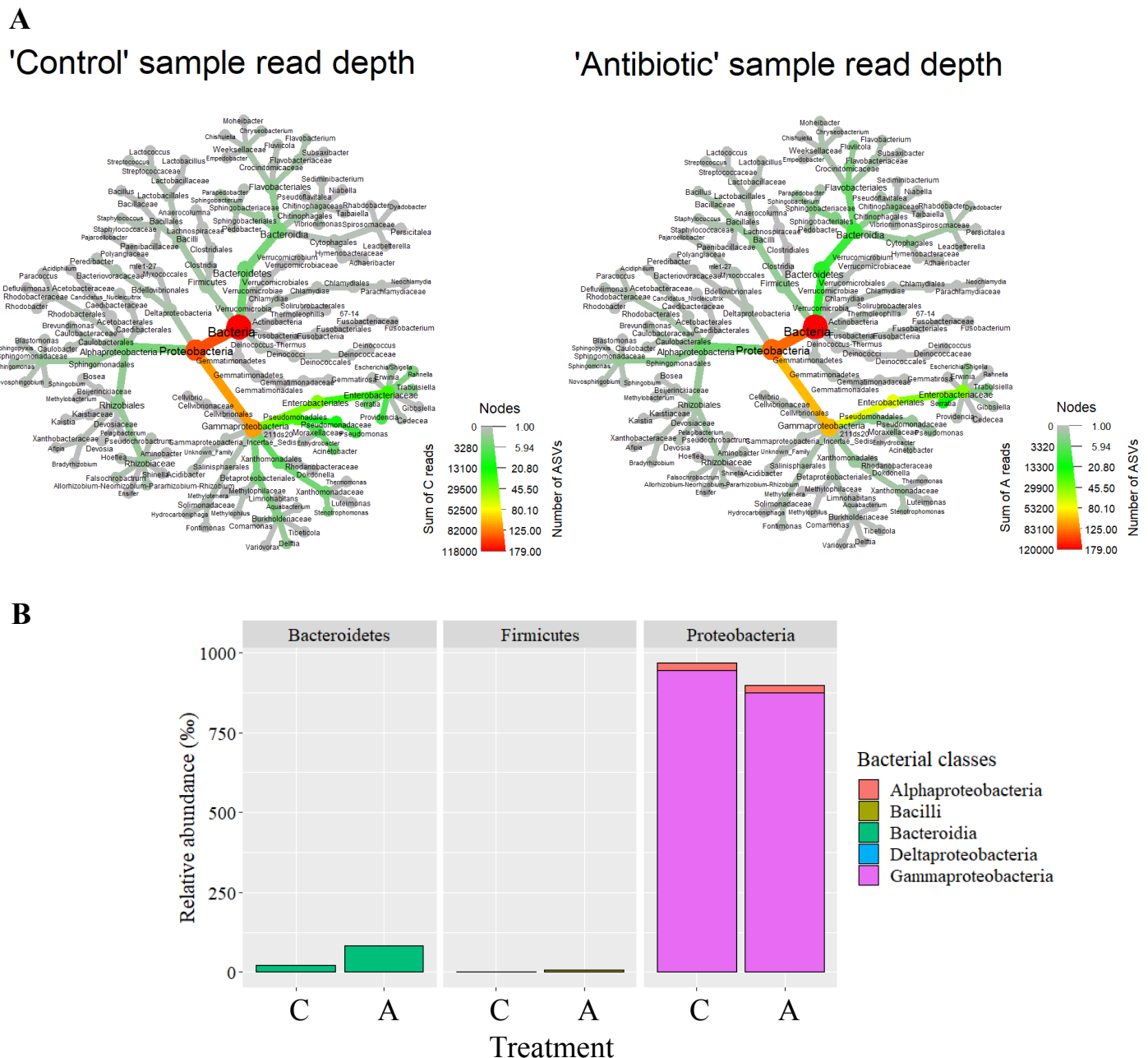
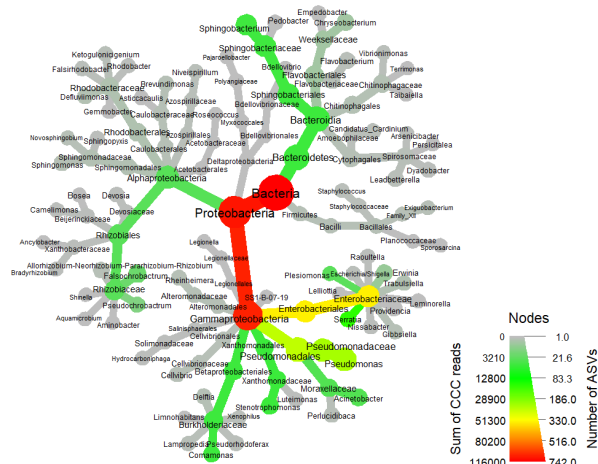


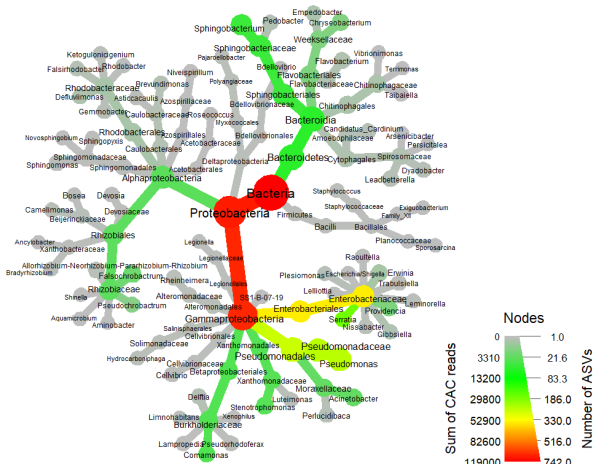
Figure S1 Dominant identified bacterial taxa of G0 flies. A) Heat trees corresponding to the sample read depth after the rarefying step for the antibiotic and control treatment of G0 flies. B) Barplot of the relative abundance (from rarefied, normalized and filtered data) of the dominant bacterial phyla and classes. "C" and "A" stand for control and antibiotic treatments respectively.

A

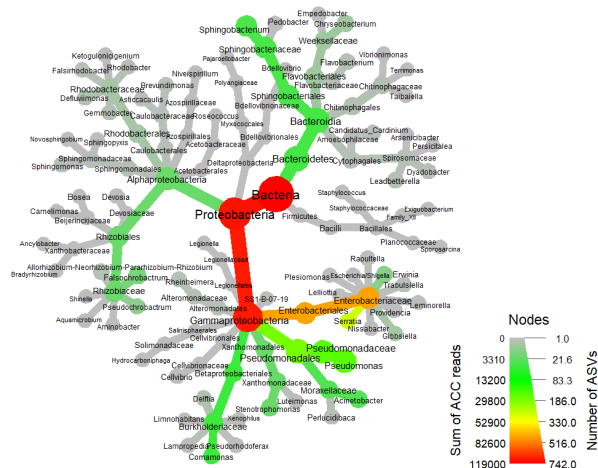
'G2-CCC' sample read depth



'G2-CAC' sample read depth



'G2-ACC' sample read depth



'G2-AAC' sample read depth

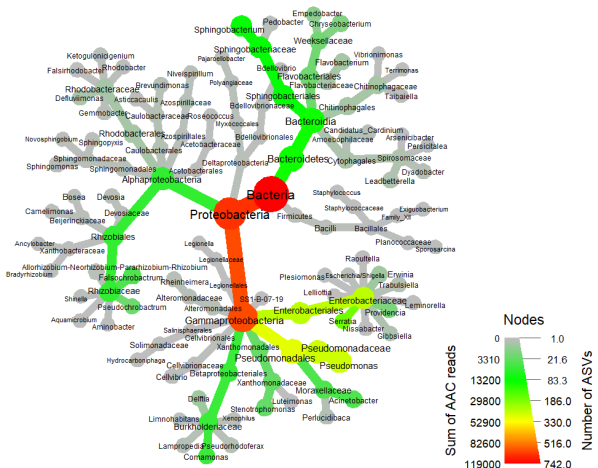
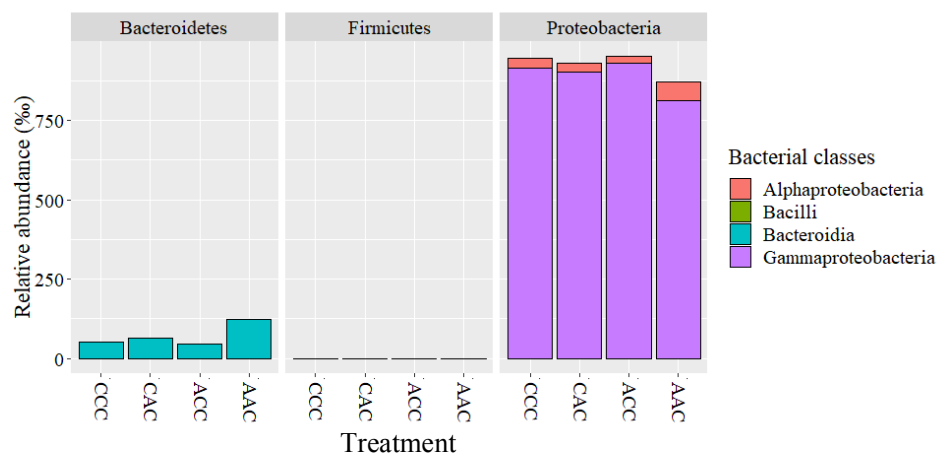
**B**

Figure S2 Dominant identified bacterial taxa of G2 flies. A) Heat trees corresponding to the sample read depth after the rarefying step for the antibiotic and control treatment of G2 flies. B) Barplot of the relative abundance (from rarefied, normalized and filtered data) of the dominant bacterial phyla and classes. “C” and “A” stand for control and antibiotic treatments respectively.

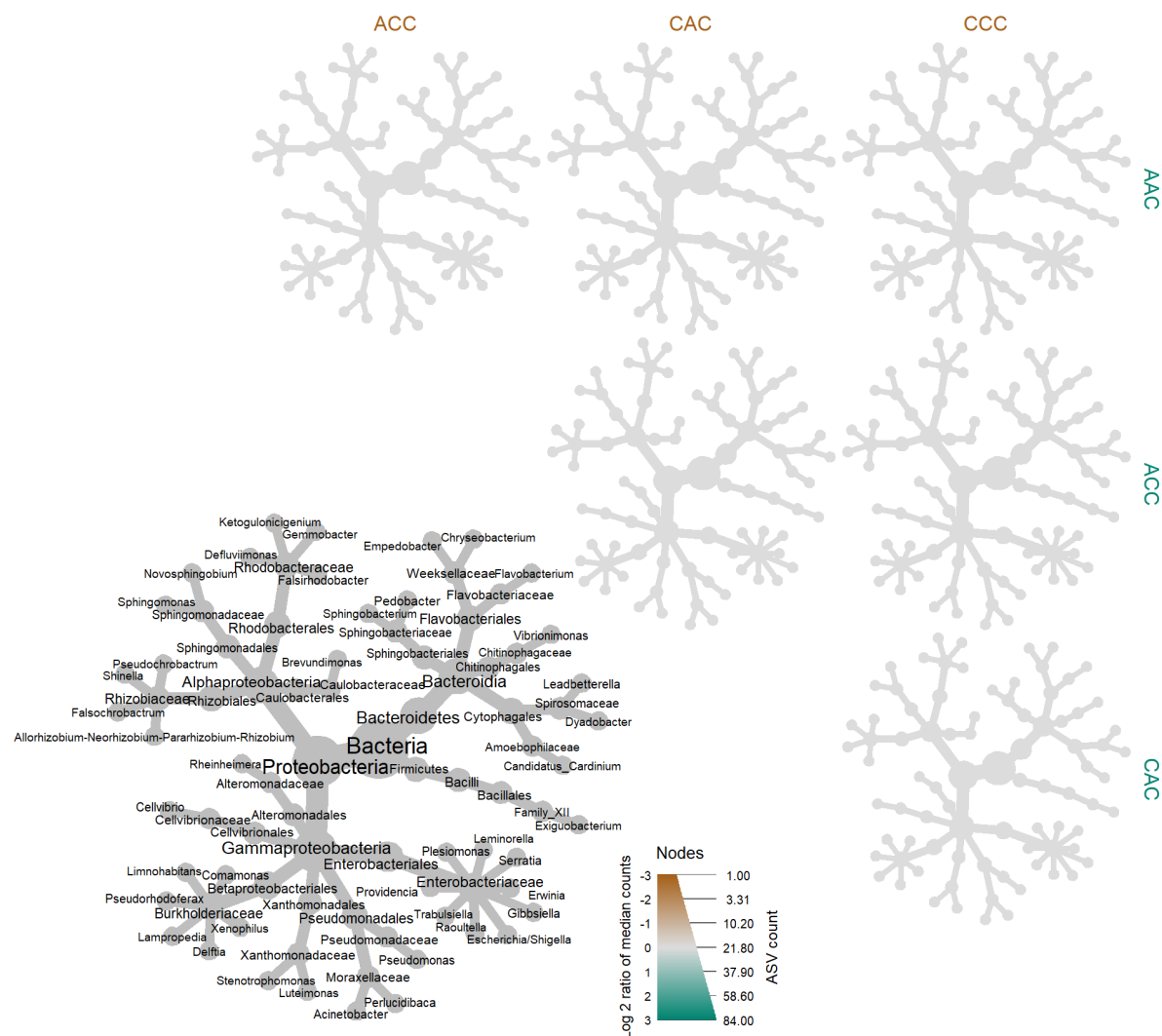


Figure S3 Heat trees comparing taxa between the antibiotic and control treatment of G2 flies. The color of each taxon represents the log₂ ratio of median relative abundances observed for each treatment (AAC, ACC, CAC, CCC) and only significant differences are colored, determined using a Wilcoxon rank-sum test followed by a Benjamini-Hochberg (FDR) correction for multiple comparisons. Taxa colored green are enriched in the treatment shown in the row and those colored brown are enriched in the treatment shown in the column. The gray tree on the lower left functions as a key for the smaller unlabeled trees.

Table S1 Statistical outputs of all GLM-analyzed ASVs associated to the G0 flies. The below table compiles taxonomic, beta diversity, mean relative abundances (% \pm se), frequencies and statistic information. Green and red colors correspond to ASV statistically more frequent in the control (C) and antibiotic (A) treatments respectively, while the grey color shows the first 100 dominant ASVs. Bold writing corresponds to ASVs significantly influenced by the treatment.

“.”: $0.1 < P < 0.05$; “*”: $0.05 < P < 0.01$; “**”: $0.01 < P < 0.001$; “***”: $P < 0.001$

<https://drive.google.com/open?id=1QcXDvkUJhfQgAkmCDkTMNHoc7LuQohsS>

Table S2 Presence and absence (i.e. 1 or 0 respectively) of bacterial genera in flies of C and A treatments. Green and red colors correspond to genera exclusively present in C or A treatments respectively.

Genus	A	C	Genus	A	C
<i>Acidibacter</i>	1	1	<i>Lactococcus</i>	0	1
<i>Acidiphilium</i>	1	0	<i>Leadbetterella</i>	1	0
<i>Acinetobacter</i>	1	1	<i>Limnohabitans</i>	1	1
<i>Adhaeribacter</i>	1	0	<i>Luteimonas</i>	1	1
<i>Afipia</i>	0	1	<i>Methylobacterium</i>	1	0
<i>Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium</i>	1	1	<i>Methylophilus</i>	0	1
<i>Aminobacter</i>	1	0	<i>Methylothera</i>	0	1
<i>Anaerocolumna</i>	0	1	<i>Moheibacter</i>	1	1
<i>Aquabacterium</i>	0	1	NA	1	1
<i>Bacillus</i>	1	1	<i>Neochlamydia</i>	1	0
<i>Blastomonas</i>	0	1	<i>Niabella</i>	1	0
<i>Bosea</i>	1	0	<i>Novosphingobium</i>	1	1
<i>Bradyrhizobium</i>	1	1	<i>Pajaroellobacter</i>	1	1
<i>Brevundimonas</i>	1	1	<i>Paracoccus</i>	1	1
<i>Candidatus_Nucleicultrix</i>	0	1	<i>Parapedobacter</i>	1	1
<i>Caulobacter</i>	1	1	<i>Pedobacter</i>	1	1
<i>Cedecea</i>	0	1	<i>Pelagibacterium</i>	1	0
<i>Cellvibrio</i>	1	0	<i>Peredibacter</i>	1	1
<i>Chishuiella</i>	0	1	<i>Persicitalea</i>	1	0
<i>Chryseobacterium</i>	1	1	<i>Providencia</i>	1	1
<i>Comamonas</i>	0	1	<i>Pseudochrobactrum</i>	1	1
<i>Defluviimonas</i>	1	0	<i>Pseudoflavitalea</i>	1	1
<i>Deinococcus</i>	0	1	<i>Pseudomonas</i>	1	1
<i>Delftia</i>	1	1	<i>Rahnella</i>	1	1
<i>Devosia</i>	1	0	<i>Rhabdobacter</i>	1	0
<i>Dokdonella</i>	1	1	<i>Rhodobacter</i>	1	1
<i>Dyadobacter</i>	1	0	<i>Sediminibacterium</i>	1	1
<i>Empedobacter</i>	1	0	<i>Serratia</i>	1	1
<i>Enhydrobacter</i>	0	1	<i>Shinella</i>	0	1
<i>Ensifer</i>	1	0	<i>Sphingobacterium</i>	1	1
<i>Erwinia</i>	1	1	<i>Sphingobium</i>	1	1
<i>Escherichia/Shigella</i>	1	1	<i>Sphingomonas</i>	1	1
<i>Falsochrobactrum</i>	1	1	<i>Sphingopyxis</i>	1	1
<i>Flavobacterium</i>	1	1	<i>Staphylococcus</i>	1	1
<i>Fluviicola</i>	1	1	<i>Stenotrophomonas</i>	1	1
<i>Fontimonas</i>	1	1	<i>Streptococcus</i>	1	1
<i>Fusobacterium</i>	1	0	<i>Subsaxibacter</i>	1	0
<i>Gemmatirosa</i>	1	0	<i>Taibaiella</i>	1	1
<i>Gibbsiella</i>	1	0	<i>Thermomonas</i>	1	0
<i>Hoeflea</i>	0	1	<i>Tibeticola</i>	1	1
<i>Hydrocarboniphaga</i>	0	1	<i>Trabulsiella</i>	1	1
<i>Kaistia</i>	1	0	<i>Variovorax</i>	1	0
<i>Lactobacillus</i>	1	0	<i>Verrucomicrobium</i>	1	0
			<i>Vibrionimonas</i>	1	1
			Total	71	61

Table S3 Statistical outputs of all GLM-analyzed ASVs associated to the G2 flies. The below table compiles taxonomic, beta diversity, mean relative abundances (% \pm se), frequencies and statistic information. Green and red colors correspond to ASV statistically more frequent in the control (CCC) and antibiotic (AAC, ACC, CAC) treatments respectively, while the grey color shows the first 100 dominant ASVs. Bold writing corresponds to ASVs significantly influenced by the treatment while letters represent statistical differences between the ASV frequency of the four treatments (AAC, ACC, CAC, CCC).

“.”: $0.1 < P < 0.05$; “*”: $0.05 < P < 0.01$; “**”: $0.01 < P < 0.001$; “***”: $P < 0.001$

https://drive.google.com/open?id=1tKGihUgtCDxj0G0Ak_N8lTMsBY0kexrU

Table S4 Presence and absence (i.e. 1 or 0 respectively) of bacterial genera in flies of CCC, CAC, ACC and AAC treatments. Green and red colors correspond to genera exclusively present in CCC or CAC/ACC/AAC treatments respectively. Grey indicates genera present in all treatments.

Genus	AAC	ACC	CAC	CCC	Genus	AAC	ACC	CAC	CCC
<i>Acinetobacter</i>	1	1	1	1	<i>Leminorella</i>	0	0	1	0
<i>Allorhizobium-</i>									
<i>Neorhizobium-</i>					<i>Limnohabitans</i>	0	0	1	0
<i>Pararhizobium-</i>	0	1	1	1					
<i>Rhizobium</i>									
<i>Brevundimonas</i>	1	1	1	1	<i>Luteimonas</i>	0	0	1	0
<i>Candidatus_Cardinium</i>	1	0	1	0	<i>NA</i>	1	1	1	1
<i>Cellvibrio</i>	0	0	0	1	<i>Novosphingobium</i>	0	0	0	1
<i>Chryseobacterium</i>	1	1	1	1	<i>Pedobacter</i>	0	0	1	0
<i>Comamonas</i>	1	1	1	1	<i>Perlucidibaca</i>	1	1	1	0
<i>Defluviimonas</i>	1	1	1	1	<i>Plesiomonas</i>	0	0	1	1
<i>Delftia</i>	1	1	1	1	<i>Providencia</i>	1	0	1	0
<i>Dyadobacter</i>	1	1	1	0	<i>Pseudochrobactrum</i>	1	1	1	1
<i>Empedobacter</i>	1	0	1	0	<i>Pseudomonas</i>	1	1	1	1
<i>Erwinia</i>	1	1	1	1	<i>Pseudorhodoferax</i>	1	0	0	0
<i>Escherichia/Shigella</i>	1	0	0	1	<i>Raoultella</i>	0	1	0	0
<i>Exiguobacterium</i>	0	0	0	1	<i>Rheinheimera</i>	1	1	1	1
<i>Falsirhodobacter</i>	0	1	1	0	<i>Serratia</i>	1	1	1	1
<i>Falsochrobactrum</i>	1	1	1	1	<i>Shinella</i>	1	0	0	0
<i>Flavobacterium</i>	1	1	1	1	<i>Sphingobacterium</i>	1	1	1	1
<i>Gemmobacter</i>	1	1	1	0	<i>Sphingomonas</i>	0	0	0	1
<i>Gibbsiella</i>	1	1	1	1	<i>Stenotrophomonas</i>	1	1	1	1
<i>Ketogulonicigenium</i>	1	0	0	1	<i>Trabulsiella</i>	1	1	1	1
<i>Lampropedia</i>	1	1	0	1	<i>Vibrionimonas</i>	0	0	0	1
<i>Leadbetterella</i>	1	1	1	1	<i>Xenophilus</i>	0	0	0	1
Total						30	26	32	30

Paper 4

Bacterial heritability and acquisition in a rhizophagous insect

In preparation

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Author contributions

MO, VL, DC, CM and AMC conceived and designed research. MO and VL carried out the experiment. MO performed the DNA extraction, and the library preparation with CB. MO did the bioinformatical and statistical analyses. MO wrote the manuscript that was commented and approved by all authors.

Abstract:

Insects harbor microorganisms that can play an important role in the fitness of their insect host. Several studies demonstrated the capacity of insect microorganisms to manipulate, disturb or suppress the plant response to insect herbivory. In insects, microorganisms can be transmitted from the parents (i.e. vertical transfer), from the environment where soil and plant can be microbial reservoirs for insect (i.e. horizontal transfer) or from both. The cabbage root fly (*Delia radicum*) is a root herbivore as a larva and partly develops in the soil. Our study aimed at assessing the bacterial communities throughout the development of *D. radicum* and consequently at evaluating both vertical and horizontal bacterial transmission in *D. radicum* using a high resolution metabarcoding approach based on gyrB primers.

To do so, we compared several insect development stages to assess vertical transmission and insect stages with the interacting plant compartment to assess horizontal transmission.

Our study showed that bacterial composition varied across the development stages of *D. radicum* and that several bacterial species are assumed to be vertically transmitted from the females to their eggs (*S. marcescens* and *A. guillouiae*) and potentially horizontally acquired

from the plant roots and soil (*Pseudomonas* and *L. cauensis*). Interestingly, *Serratia*, *Acinetobacter* and *Pseudomonas* genera were also identified in the literature as carrying a gene responsible for plant defense detoxification.

To go further, studies should move on toward functional approaches to understand the role of insect symbionts in plant-insect interactions, by coupling metabarcoding to prediction tools.

Keywords: cabbage root fly, *Delia radicum*, oilseed rape, *Brassica napus*, rhizosphere and root bacteria, vertical and horizontal transmission, *Serratia marcescens*, *Acinetobacter guillouiae*, *Pseudomonas*.

1. Introduction

Symbiosis is a neutral term describing the interaction occurring between two distinct living organisms, which nature is variable and can range from parasitism to mutualism (Sapp, 2010). Symbiosis is quite studied in insects as it has been largely demonstrated that symbiotic microorganisms can greatly impact their insect host phenotype. Indeed, it was shown that symbionts can provide a better resistance to heat shock or fecundity to their aphid hosts (Guo *et al.*, 2017), and even protection against natural enemies like parasitoids, nematodes or pathogens (Oliver *et al.*, 2014). Moreover, an increasing number of studies has highlighted the capacity of insect symbionts to manipulate, disturb or suppress the plant response to insect herbivory (Zhu *et al.*, 2014). For example, leaf mining caterpillars are able to increase their nutritional intake with their endosymbiotic bacteria modifying the plant phytohormones (Kaiser *et al.*, 2010; Body *et al.*, 2013). The coffee berry borer is another example of plant manipulation through symbionts, where gut *Pseudomonas* strains are able to degrade tannic defenses produced by coffee plants, so that the seeds would be eatable for their host (Ceja-Navarro *et al.*, 2015). However, the way insect symbionts are transmitted is not always known.

Transmission of symbionts is variable as they can be acquired either from the parents (i.e. vertical transmission), the environment (i.e. horizontal transmission; which encompasses the soil, water, air or other organisms), or from both (Frago *et al.*, 2012).

Regarding vertical transmission, it is largely admitted for insects that the mother can transmit symbionts through colonization of the eggs or embryos (Moran and Dunbar, 2006), with females coating the egg shell with microorganisms from their reproductive organ for example (Bakula, 1969). For example, the stinkbug (*Megacopta punctatissima*), produces both eggs and small brownish particles containing the obligate symbiont, which is orally and externally inherited by the hatchlings that feed on the particles (Hosokawa *et al.*, 2007). Transmission can also occur internally like for the symbiont *Buchnera*, which is transmitted to the embryos within the female ovaries (Michalik *et al.*, 2014).

Concerning horizontal transmissions, symbiont acquisition can occur through coprophagy, with newly born offspring that obtain symbionts by probing on conspecific feces (Salem *et al.*, 2015), through predation and cannibalism (Le Clec'h *et al.*, 2013) or even parasitism with parasitoids (Qi *et al.*, 2019). Diet and water can also be used to transfer or modify gut microbiota (Mason *et al.*, 2014). Additionally, recent studies highlighted plant-

mediated horizontal transmission where insect symbionts can be passed down to plants, through direct injection from phloem-sucking aphids or through feces and honeydew-contaminated surfaces, which in turn can infect other insects feeding on the plant (Chrostek *et al.*, 2017). In some cases, newly acquired symbionts have a successful establishment and can be vertically transmitted to insect offspring. For example, a part of the microbiota of the caterpillar *Mamestra brassicae* seems to be horizontally transmitted, as similarities were found between its microbiota and the one from leaves and soil (Hannula *et al.*, 2019). It also has been demonstrated that specific and identified microorganisms inoculated into the soil can be taken up by the plant and later on transmitted to the insects feeding on the plant, like *Serratia symbiotica* acquired by aphids (Pons *et al.*, 2019) or *Bacillus thuringiensis* acquired by the cabbage moth or the fall armyworm (Monnerat *et al.*, 2009). The soil is a very well-known microbial reservoir for plants (Vandenkoornhuyse *et al.*, 2015), but less recognized for insects while it can also be important. For example, the bean bug *Riptortus pedestris* acquires its symbiotic bacteria of the genus *Burkholderia* from the soil during its development and this acquisition improves its resistance to insecticides (Kikuchi *et al.*, 2012). While many studies have highlighted symbiont transmission in aboveground insects, few have focused on belowground herbivores.

The cabbage root fly (*Delia radicum*) is a root herbivore of Brassicaceous plants as a larva and several studies have characterized *D. radicum* bacterial communities at different stages but using different methodologies. Lukwinski *et al.* (2006) has detected cultivable bacteria at the egg stage, identified several bacterial species (*Pseudomonas*, *Erwinia* and *Serratia*) from Enterobacteriaceae family (i.e. from of γ -Proteobacteria class) in the larval gut, and observed hydrolysis activities in the gut associated to several groups of bacteria. Similarly, Welte *et al.* (2016) showed that several strains of γ -Proteobacteria possessed plasmids containing a *saxA* gene responsible for the degradation of toxic plant defenses. Additionally, the adult stage was described by Bili *et al.* (2016), where two *D. radicum* populations shared 4 bacterial genera (i.e. from the α - and β -Proteobacteria classes and Tenericutes phylum), with *Wolbachia* largely dominating the bacterial communities. The presence of *Wolbachia* may have biased the observed bacterial diversity, as it has been shown that *Wolbachia* could impoverish insect bacterial communities (Zouache *et al.*, 2009). Doane and Chapman (1964) were the first to initiate study about vertical and horizontal transmission of microorganisms in *D. radicum*. Relying on the assessment of appearance and growth characteristics of microorganisms through a cultivable approach, the authors showed that there were similarities between eggs and pupae and between larvae and plant roots. Later on, the study of Flury *et al.* (2018) used fluorescence

microscopy to show that *D. radicum* larvae and adults acquired *Pseudomonas protegens* from plants and then served as a vector to spread the bacteria to other plants. Thus, knowledge about the vertical and horizontal microbial transmission in *D. radicum* is still very limited.

Our study aimed at assessing *D. radicum* bacterial communities throughout its development and consequently evaluating both vertical and horizontal transmission of bacteria, using a high resolution metabarcoding approach based on *gyrB* primers. To get a better view of other components of the symbiotic bacterial community, we used a *Wolbachia*-free population. We also tried to answer the following questions: Are there bacteria inside eggs? Can bacteria be transmitted from one insect development stage to another? Are bacterial communities similar between a development stage of two different generations? Are there shared bacteria between the insect and its host plant? For that purpose, the bacterial communities of two egg generations, developing larvae, pupa shells, adults and host plant root and rhizosphere were compared. Alpha and beta diversities were analyzed and comparisons between stages or compartments were performed using generalized linear models and a newly-developed and innovative statistical plotting analysis called “differential heat trees” (Foster *et al.*, 2017).

2. Materials and Methods

2.1. Plant growth

The soil used in our experiment was collected in August 2016, from the layer –10 to –30 cm deep of a field in Brittany (La Gruche, Le Rheu, France, 48°08'44"N, 01°47'97" W) where wheat was cultivated for 20 years. It was stored outside under a tarpaulin for a year before being mixed to 1/3 volume of silica. Seeds of *Brassica napus* L. (subsp. *oleifera* cv. Tenor) were sown in individual pots using a layer of pozzolan at the bottom and the silica-soil mixture. Plants were watered twice a week by sub-irrigation with a nutritive solution based on Hoagland and Arnon (1950) during the whole experiment. This solution was obtained by blending three separate solutions: a macronutrient solution (3 mM of KNO₃, 0.5 mM of KH₂PO₄, 1 mM of MgSO₄ 7H₂O, 2.5 mM of Ca(NO₃)₂ 4H₂O), a micronutrient solution (10 µM of MnSO₄ H₂O, 1 µM of ZnSO₄ 7H₂O, 0.5 µM of CuSO₄, 30 µM of H₃BO₃, 1 µM of Na₂MoO₄ 2H₂O, 0.5 µM of Co(NO₃)₂ 6H₂O), and a Fe EDTA solution (27 µM of Fe EDTA Na). Plants were cultivated in a climate cabinet (photoperiod 16:8 LD and thermoperiod 20:18°C LD) for seven weeks before the start of the experiment.

2.2. Insect rearing

The population of cabbage root fly (“CRF”, *Delia radicum*) used in our experiment was collected in a field in Pleumeur-Gautier (Brittany, France) during 2016 and was characterized by an absence of the *Wolbachia* endosymbiont (0%; Lopez, 2018). In our laboratory, the flies got water from wet cotton and were fed on a mixture composed of sugar, dried milk and yeast (ratio 1:1:1) and were reared on rutabaga roots (*Brassica napus* subsp. *rapifera*) in a climatic room (16:8 LD, $21 \pm 2^\circ\text{C}$; $60\% \pm 10\%$ RH). In field conditions, females lay their eggs (i.e. the oviposition) at the base of plant stems, also called the crown. Hatching occurs within a few days and the three larval instars feed by tunneling inside the roots for 2 to 3 weeks before pupating in the nearby soil. Adults emerge about a month after oviposition and can live up to 45 days when supplied with food and water (Finch and Coaker, 1969).

2.3. Experimental setup and sample collection

Adult flies were left to oviposit on a piece of bleached rutabaga roots, resting on a sterile filter in a Petri dish with the overall placed in a cage, for 72h in order to obtain black-headed eggs (i.e. ready to hatch). Infestation by *D. radicum* was performed by depositing 10 black-headed eggs at the plant stem base (figure 1).

Different development stages of the CRF were sampled: female and male adults (first generation, “G”), they produced another generation of eggs (“G+1”), which also served to infest the plant, fed larvae, 24 hour-starved larvae (expected to have less plant materials in the gut than fed larvae and thus more representative gut bacterial communities associated to the insect), male and female empty pupae and another generation of eggs (G+2).

Regarding the plant, the two organs were collected at the same time as each CRF egg (G) and larval stages. The roots (i.e. area from the stem base to the root tips) were cut into small pieces. The rhizosphere (i.e. the soil layer attached to the roots) was obtained through two baths of 20 mL sterile permuted water, followed by a 20 min centrifugation at 5,000 g and at 4°C and the supernatant removal. The two collected organs were immediately stored at -80°C until further use.

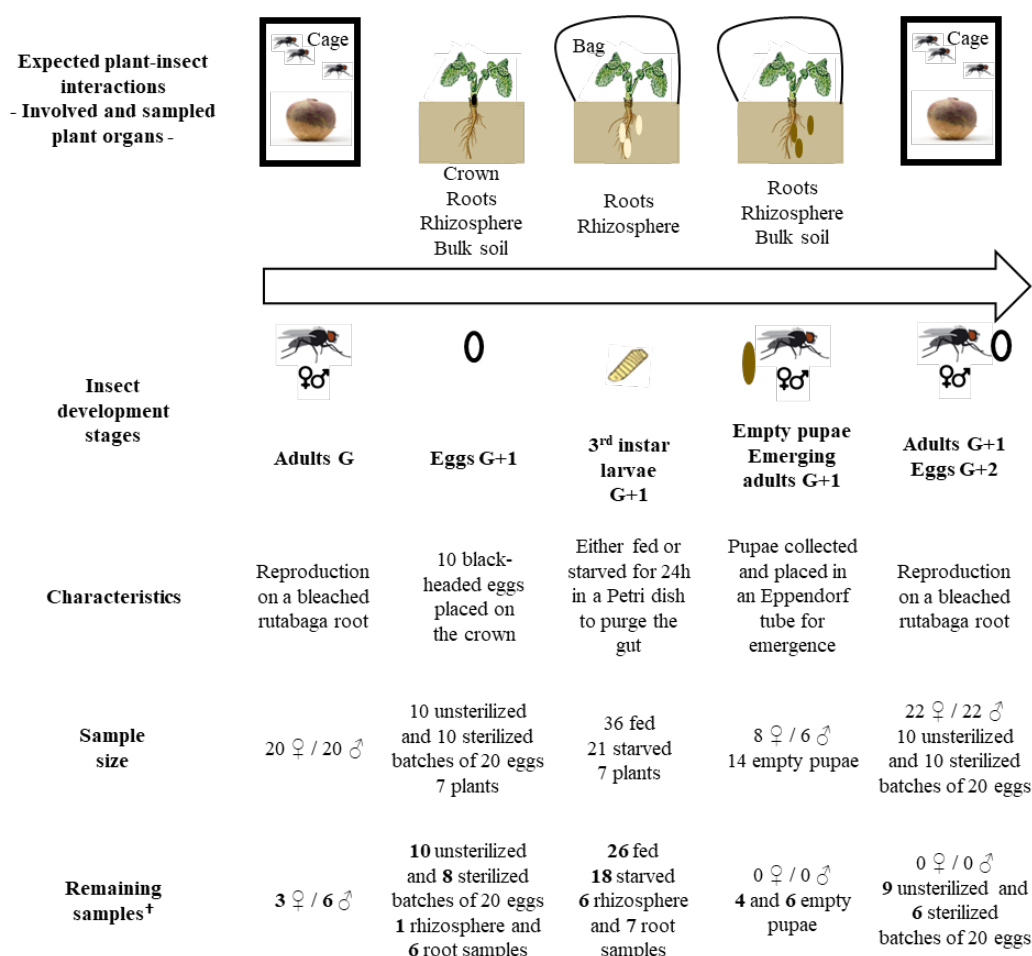


Figure 1 Experimental design. Plant and insect samples were collected according to the different development stages of *D. radicum*.

[†]Number of samples that remained after the bioinformatical analysis and the cleaning steps (rarefying, normalizing and filtering steps).

2.4. Molecular analysis

2.4.1. Rhizosphere soil samples

Rhizosphere samples collected during the experiment, were freeze-dried and then analyzed. Rhizosphere soil DNA was extracted based on the protocol developed by the GenoSol platform (Dijon, France) and adapted from Plassart *et al.* (2012) and Ourry *et al.* (2018). One gram of freeze-dried rhizosphere soil was added to a 15 mL lysing matrix E tube (MP Biomedicals, Santa Ana, California, USA), which contained 1.4 mm ceramic spheres, 0.1 mm silica spheres, and eight 4 mm glass beads. Then, to the same tube we added 5 mL of lysis buffer containing 100 mM of Tris-HCl (pH 8), 100 mM of EDTA (pH 8), 100 mM of NaCl, 2% SDS and sterile ultrapure water. In order to avoid plug formation, tubes were manually agitated and agitated in a FastPrep ®24 (MP Biomedicals, Santa Ana, California, USA) at 4 m.s⁻¹ during 90 s (3 times 30 s). They were rapidly vortexed and incubated in a bath at 70°C for

30 min, during which samples were again vortexed at mid and at the end of incubation. Tubes were centrifuged at 3,500 rpm at 15°C for 10 min and supernatant was transferred to a Nalgene cryotube for further analysis. For the next steps of the DNA extraction, samples were duplicated in order to obtain a higher DNA concentration. Deproteinization was performed by adding 1/10 volume of 3 M potassium acetate (pH 5.5) to 1 mL of lysate (2 tubes per sample), then the tubes were homogenized by turnaround and incubated for 10 min on ice, before being centrifuged at 14,000 g at 4°C during 10 min. For DNA precipitation, approximately 900 µL of the supernatant was transferred to a clean tube, where 900 µL of ice-cold 100% isopropanol were added and tubes were agitated by turnaround, then placed at -20°C for a night. To obtain DNA pellets, tubes were centrifuged at 13,000 rpm at 4°C for 30 min and supernatant was discarded. DNA pellets were washed as follows: 400 µL of 70% ice-cold ethanol were added to samples, which were centrifuged at 13,000 rpm at 4°C for 5 min and supernatants were removed. In order to eliminate remaining traces of ethanol, unlidded tubes were placed at 60°C for at least 15 min or more if needed. Pellets of DNA were resuspended with 100 µL of sterile ultrapure water and the duplicated samples were finally pooled.

Rhizosphere samples were purified twice. The first purification required Microbiospin (Biorad, Hercules, California, USA) columns of PVPP (PolyVinyl PolyPirrolidone, Sigma-Aldrich), which were prepared according to the protocol described in Ourry *et al.* (2018). One hundred microliters of DNA were injected into the columns, previously transferred to a clean tube, and samples were incubated on ice for 5 min, before a 4 min centrifugation at 1,000 g at 10°C. The obtained DNA (approximately 95 µL) was used for the second purification, performed using the GeneClean® Turbo kit (MP Biomedicals). Five volumes of GTGNSS (GeneClean Turbo GNomic Salt Solution) were added to the obtained DNA and samples were homogenized by pipetting. The whole mixture was injected in purification columns from the kit, which were centrifuged at 10,000 g for 10 s at 10°C and tubes were emptied. Then, 500 µL of GTW (GeneClean Turbo Wash) were injected in columns and tubes were again centrifuged and emptied, this whole step was carried out a second time. Empty columns were centrifuged at 10,000 g during 4 min at 10°C, before injecting 30 µL of GTE (GeneClean Turbo Elution Solution). Samples were incubated on ice for 5 min and centrifuged at 10,000 g for 1 min at 10°C, then the GTE, incubation and centrifugation steps were repeated a second time to finally obtain approximately 60 µL of clean DNA. Samples were then stored at -20°C until further use.

2.4.2. Root samples

Root samples were freeze-dried and ground using glass beads. DNA was extracted using the NucleoSpin® Plant II kit and protocol (Macherey-Nagel, Düren, Germany). The procedure is available on the manufacturer website, but we made some modifications. Extraction was performed on 30 mg of freeze-dried and ground matter, to which were added 400 µL of lysis buffer PL1 for cell lysis before thoroughly vortexing the tubes to eliminate the aggregation that followed. Ten microliters of RNase A was added before a new vortexing phase and then samples were incubated in a water bath at 65°C for 30 min. The obtained crude lysate was centrifuged for 5 min at 11,000 g and at 17°C, then the supernatant was transferred into a Nucleospin Filter, placed on a new tube, before a 2 min centrifugation. The collected clear flow-through was mixed to 450 µL of binding buffer PC by vortexing and 700 µL of this mixture was transferred to a Nucleospin Plant II column, then centrifuged for 1 min. Tubes were emptied and the column was alternatively washed and centrifuged several times with 400 µL of wash buffer PW1 for 1 min, with 700 µL of wash buffer PW2 for 1 min and with 200 µL of PW2 for 2 min. Each time, the tubes were emptied. Then, the column was transferred into a new tube and 50 µL of the warmed elution buffer PE were added before incubating the samples in a water bath at 65°C for 5 min and centrifuging for 1 min. This elution step was repeated a second time. Samples were then stored at –20°C until further use.

2.4.3. Insect samples

Upon collection and prior to analysis, individuals of *D. radicum* were stored in 96% ethanol at –20°C. In order to analyze the inner microbiota of each development stage, larvae and half of the egg samples were sterilized with 3.2% bleach during 1 min. Bleached samples were then rinsed three times with sterile ultrapure water during 1 min and dried out on a sterilized filter before being placed in a semi deep well plate. The other half of the egg samples remained unsterilized to evaluate potential maternal contamination.

DNA was extracted using 300 µL of lysis buffer: 1 M of Tris, 5 M of NaCl, 0.5 M of EDTA, 20% SDS and sterile ultrapure water; 6 µL of proteinase K (5 mg/mL) and 3 sterilized glass beads (3 mm diameter) added to each well. The plate was sealed and samples were ground during 6 min and incubated at 37°C overnight. Then, plates were rapidly centrifuged and 85 µL of NaCl (5 M) were added per well, followed by short vortexing and a 30 min centrifugation at 3,500 rpm and 4°C. Approximately 290 µL of supernatant were then transferred to a new plate containing 280 µL of 100% ice-cold ethanol and samples were homogenized by pipetting and incubated at –20°C for 1h. After a 30 min centrifugation at 3,500 rpm and 4°C, supernatant was

discarded and pellet was washed with 200 μ L of 70% ice-cold ethanol. After another centrifugation and elimination of the supernatant, samples were vacuum-dried during 30 min at 30°C. Pellets of DNA were resuspended in 50 μ L of sterile ultrapure water, but only in 30 μ L for egg and empty pupae samples, and stored at 4°C overnight. Samples were then transferred at -20°C until further analysis.

2.5. Amplicon library construction and sequencing

Samples were displayed in several plates. A first PCR was performed on 2 μ L of the obtained DNA using the *gyrB*_aF64/ *gyrB*_aR353 primer pair (5'-MGNCCNGSNATGTAYATHGG-3'/5'-ACNCCRTGNARDCCDCCNGA-3') that amplifies *gyrB* the subunit B of the bacterial gyrase (Barret *et al.*, 2015), which was combined to a tag for plate multiplexing and an Illumina index attachment. For the following steps of PCRs and purifications, the protocol was modified from Bartoli *et al.* (2018). A final volume of 25 μ L was reached by adding 23 μ L of a master mix containing 2.5 μ L of MTP taq Buffer (SIGMA), 0.5 μ L of 10 mM dNTPs, 1 μ L of the forward primer (10 p/mol), 1 μ L of the reverse primer (10 p/mol), 0.2 of DMSO, 0.5 μ L of bovine serum albumin (BSA), 0.2 of MTP taq DNA polymerase (SIGMA, ref. D7442-1500UN) and 17.1 μ L of sterile ultrapure water. DNA was denatured at 95°C for 5 min, followed by 40 cycles of amplification at 95°C (30 s), 50°C (60 s) and 70°C (90 s), with a final extension step at 70°C for 10 min. Plates were then pooled by pairs with now a volume of 50 μ L per well. PCR 1 products were purified using NucleoMag® NGS Clean-up and Size Select kit (Machery-Nagel) according to the protocol of the manufacturer with the following modifications: 35 μ L of magnetic beads were used for a volume of 50 μ L per well, washing was realized with 150 μ L of 80% ethanol, DNA was eluted in 30 μ L of sterile ultrapure water. As we obtained a low DNA concentration after Nanodrop quantification, we used 8 μ L of the purified PCR 1 products to perform the second PCR, which added the Illumina index, with 42 μ L of a master mix containing 5 μ L of 10X MTP taq Buffer, 1 μ L of 10 mM dNTPs, 1.25 μ L of PCR 2 forward P5 primer (20 μ M), 1.25 μ L of PCR 2 reverse P7 index primer (20 μ M), 0.5 μ L of MTP taq DNA polymerase (SIGMA, ref. D7442-1500UN) and 39 μ L of sterile ultrapure water. DNA was then denatured at 94°C for 1 min, followed by 12 cycles of amplification at 94°C (60 s), 65°C (60 s) and 72°C (60 s), with a final extension step at 72°C for 10 min. A second purification occurred as previously described, followed by Nanodrop quantification and the purified amplicons were pooled in a 20 ng equimolar concentrations. Samples were sequenced using Illumina MiSeq to a 2 x 300 bases paired-end version at the

GeT-PlaGe platform (Toulouse, France). Raw data sets will be deposited on the European Nucleotide Archive database system.

2.6. Bioinformatics analyses

Samples were demultiplexed with Flexbar tool (Dodt *et al.*, 2012) using the barcodes, which were then trimmed, then primers were also trimmed using BBduck from the BBMap package (<https://jgi.doe.gov/data-and-tools/bbtools/>). The output was: a table containing the number of reads per sample after these two steps and two fastq files per sample respectively for read 1 and read 2. The table showed a varying number of reads per sample, which is why only samples which number of reads were higher than 1000, when looking at the read 1, were kept for further analysis.

The dada2 workflow, based on Divisive Amplicon Denoising Algorithm (“DADA”) was used with the “dada2” R package on our samples to obtain an amplicon sequence variant (ASV) table, which identified fine-scale variations compared to the operational taxonomic unit (OTU) table (Callahan *et al.*, 2016).

We made the following modifications to the default functions proposed by the dada2 workflow and package. After inspecting the quality profiles of reads 1 and 2 through plotting, trimming was performed at 200 and 150 for read 1 and read 2 respectively where the quality score started to drop below than 30. To learn the error rates, we increased the number of samples, bases and reads taken into account by the machine-learning algorithm, with the arguments “nbases = 1e+09” and “randomize = TRUE”. Then, the dereplication, sample inference and merging steps were performed as proposed in the workflow. Lastly, the sequence lengths were inspected and only sequences which length ranged from 244 and 256 nucleotides were kept, then chimeric sequences were removed.

Taxonomic affiliations were performed using the in-house *gyrB* database, 2nd version (Barret *et al.*, 2015; Bartoli *et al.*, 2018). At the end of this workflow, samples with 0 remaining reads were removed from the dataset.

2.7. Statistical analyses

Analyses were performed using the R software (R Core Team, 2018) and a 5% threshold for statistical significance.

For data manipulation, we used the “phyloseq” and “microbiome” packages (Lahti *et al.*, 2012; McMurdie and Holmes, 2013). Plant and insect samples were first analyzed

separately. First step, we compared insect development stages in order to evaluate the bacterial communities and to assess vertical bacterial transmission between the development stages. Second step, we evaluated the bacterial communities of the sampled plant compartments and then compared insect and plant bacterial communities in order to assess horizontal transmission between *D. radicum* development stage and the plant compartment the insect was interacting with.

Rarefaction curves were obtained using the “ggrare” function from the “ranacapa” package (Kandlikar, 2019) to make sure that all ASVs were detected in each sample. Then samples were rarefied using the “rarefy_even_depth” function and the setting “set.seed(400)” with a sample size of 2500 and 25,000 respectively for insect and plant samples, as maximum richness was reached at different sample size. Samples were normalized using per mille proportions (i.e. the sums of reads per sample transformed in 1000) instead of percentage as many ASVs had a very low abundance, and then filtered by removing ASV which proportions were lower than 1/1000.

Alpha diversity analysis was performed on normalized samples and both richness (i.e. number of observed ASVs) and Shannon index were calculated using the “estimate_richness” function from the “phyloseq” package. Each index was tested against the insect development stages (i.e. adults G, eggs G+1, larvae G+1, empty pupae G+1, eggs G+2) and sample characteristics (i.e. female or male, sterilized or not, fed or starved) using a linear model when analyzing insect bacterial communities and against the plant compartment (i.e. roots and rhizosphere) and insect development stages (i.e. eggs G+1, larvae G+1) when analyzing plant bacterial communities. The significance of each term in the model was determined by a F-test as a type II analysis of variance (“Anova” function, “car” package, Fox and Weisberg, 2011). When a factor was significant, the estimated marginal means (“emmeans” function, the “emmeans” package, Lenth, 2019) were calculated and a Tukey test was applied to perform pairwise comparisons using the “CLD” functions (“emmeans” package), thus to assess the differences between modalities within this factor. P values were corrected using the “False Discovery Rate” (FDR) as multiple comparisons were performed (“p.adjust” function). Plotting required the “ggplot” function from the “ggplot2” package (Wickham, 2016).

Beta diversity or community structure analysis was performed on normalized and filtered samples. To assess the transmission of bacterial species, it was necessary for ASV proportions to be turned into a 0/1 matrix, thus presence-absence table. Then, data were transformed using the Hellinger distance as it is the method used for 0/1 matrix. A

transformation-based redundancy analysis (tb-RDA) was applied on these data (“rda” function from the “vegan” package) using the insect development stages and sample characteristics for insect bacterial communities on the one hand, and plant compartment and insect development stages for plant bacterial communities on the other hand, to build the model (Oksanen *et al.*, 2016). The “RVAideMemoire” package (Hervé, 2016b) was used to perform a type II permutation F-test for constrained multivariate analyses to test the significance of each term in the model (“MVA.anova” function), pairwise comparisons when a factor was significant to assess the differences between modalities within this factor (pairwise.factorfit” function) and for plotting the data (“MVA.plot” function).

Using rarefied, normalized and filtered data, we plotted the relative abundance of bacterial phyla and classes, obtained with the “tax_glom” function (“phyloseq” package), to visually identify the dominant phyla and classes in each insect development stage and plant compartment (“ggplot” function).

In order to visualize community data at the different taxonomical level and map differences or similarities using colors and thus evaluate vertical and horizontal transmissions, we realized heat trees with the “heat_tree_matrix” function from the “metacoder” package developed by Foster *et al.* (2017). Heat trees were realized on normalized and filtered data, which were transformed into presence/absence data with an adapted function, hence replacing the default Wilcoxon test in the “compare_groups” function, so that color 1 in the heat tree corresponds to taxa present in all replicate of treatment 1, color 2 for those in treatment 2 and color 3 for taxa present in all replicates of both treatments.

Insect bacterial species, that either stood out from the previous analyses or that were identified from the literature (Lukwinski *et al.*, 2006; Bili *et al.*, 2016; Welte *et al.*, 2016) as being present in the CRF, were retained. To test whether these targeted species were spread or varied across the CRF development, we used using generalized linear model (GLM) with binomial error and a logit link function and the presence of a given species was tested against the insect development stages. A likelihood-ratio test was performed on the model to test the significance of the term in the model and then pairwise comparisons when a factor was significant to evaluate the differences between the modalities within this factor (“Anova”, “emmeans”, “CLD” functions), followed by FDR-corrected P values as multiple comparisons were performed.

3. Results

3.1. Bacterial characterization throughout insect development

After the rarefying step, a total of 7,500 and 15,000 reads were detected respectively in female and male adults G; 25,000 and 20,000 reads for unsterilized and sterilized eggs G+1; 65,000 and 45,000 reads for fed and starved larvae G+1; 10,000 and 15,000 for female and male empty pupae G+1; 22,500 and 15,000 for unsterilized and sterilized eggs G+2.

Alpha diversity was assessed by evaluating the number of observed ASVs and calculating the Shannon index (figure 2A). The number of observed ASVs was influenced by the development stages ($F=4.402$; $df=2$; $P=0.015$) and by sample characteristics ($F=3.633$; $df=3$; $P=0.015$). We observed that there were significantly more ASVs in eggs G+2 (mean of 60-77 ASVs) than eggs G+1 (mean of 29-31 ASVs), as well as in fed larvae (mean of 50 ASVs) compared to starved ones (mean of 27 ASVs). The Shannon index was also influenced by the sample characteristics ($F=5.641$; $df=3$; $P=0.0013$) but not by the development stages of *D. radicum* ($F=0.431$; $df=2$; $P=0.65$), with fed larvae (mean value of 2.95) having significantly more diverse communities than larvae that were starved for 24h (mean value of 2.30).

Beta diversity, or the bacterial community structure was driven by the insect development stage ($F=1.571$, $df=2$; $P=0.001$) and by sample characteristics ($F=1.238$; $df=3$; $P=0.001$, figure 2B). Our model explained 10.18% of the total constrained variance, with the insect development stage accounting for 3.03% and sample characteristics for 3.58 %. The bacterial community structure was significantly different between all development stages except for adults G and eggs G+1, which structures were similar. Regarding sample characteristics, there were no differences between males and females, and between fed and starved status, while unsterilized and sterilized status significantly differed in their bacterial community structures. Moreover and according to the correlation circle (figure 2B), unsterilized eggs G+1 seemed to be associated with several ASVs: ASV 10, ASV 15, ASV 33, ASV 3285, ASV 5218, respectively corresponding to *Acinetobacter guillouiae*, *Serratia marcescens*, *Bacillus thuringiensis*, an unknown Firmicute and *Agrobacterium tumefaciens* (figure 2B, table S1).

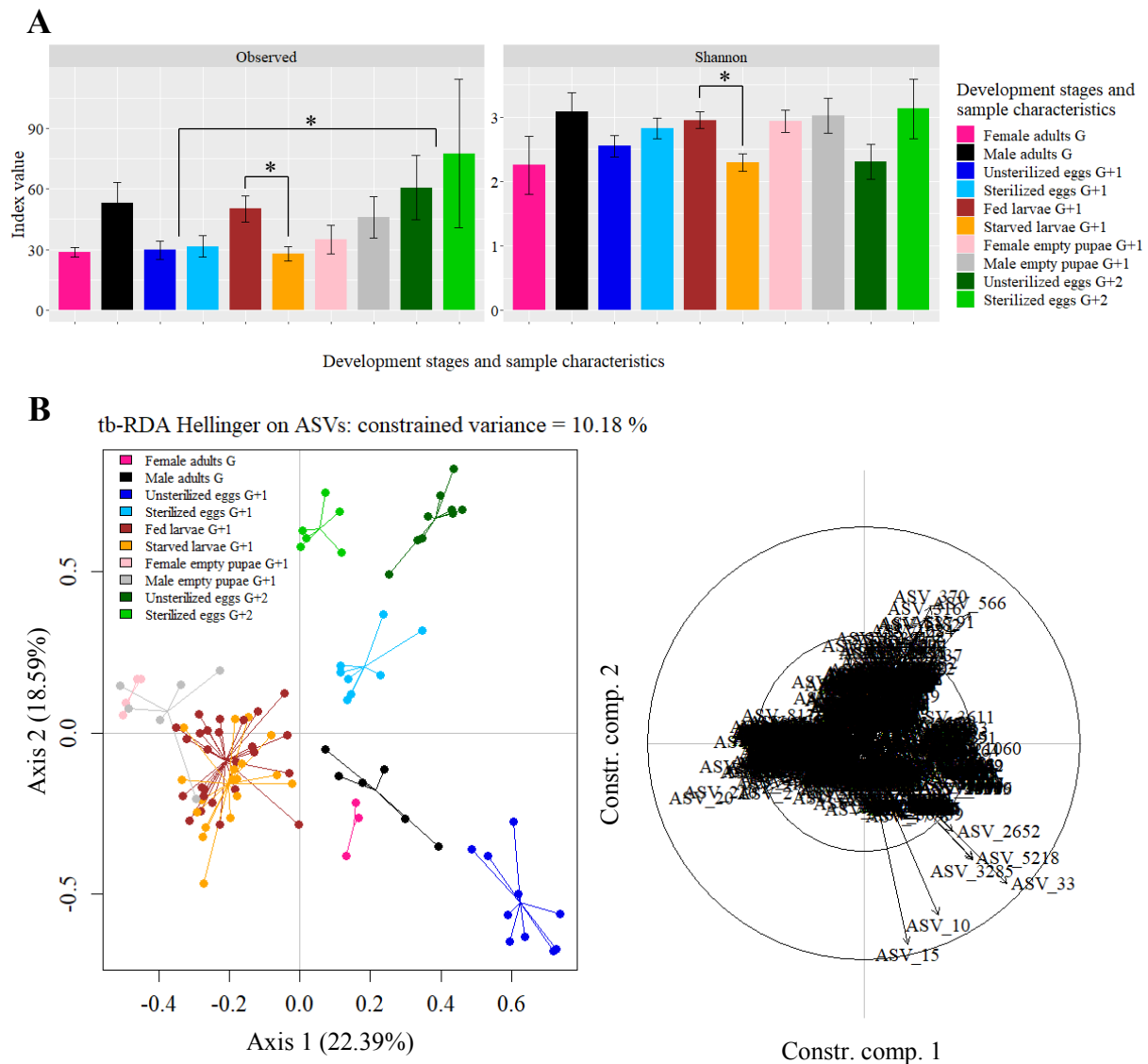


Figure 2 Bacterial diversity of the different insect development stages. A) Alpha diversity is represented by the number of observed ASVs and the Shannon index (mean \pm se). An asterisk represents significant differences between two stages or sample characteristics. B) Community structure is represented by beta diversity analyzed using a tb-RDA performed on Hellinger-transformed presence/absence data, with sample projection on the left plot and the correlation circle of ASVs on the right one. The constrained variances explained by the axes are given in parenthesis.

The bacterial communities at the different development stages of *D. radicum* were mainly composed of Proteobacteria and Bacteroidetes, the two most dominant phyla, followed by the Firmicutes, and more precisely of γ - and α -Proteobacteria, Sphingobacteriia and Bacilli classes (figure 3). Among the minor phyla, there were Actinobacteria, Cyanobacteria, Nitrospirae, Parcubacteria, unclassified (i.e. reads having similarities in the *gyrB* database but which sequences do not have a name for a given taxonomic level), Tenericutes and Verrucomicrobia (figure 3, table S2).

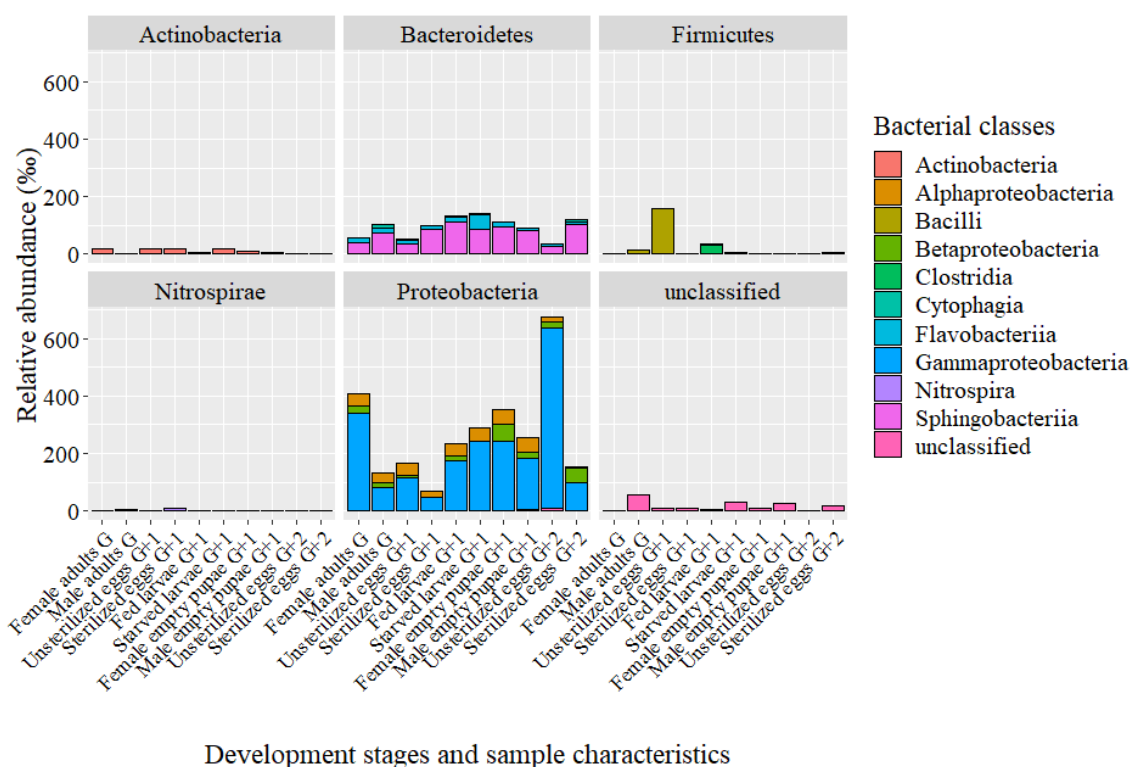


Figure 3 Bacterial phyla and classes present in the different development stages of *D. radicum*.

A total of 2466 ASVs was detected in insect samples (table S1), among which 758 ASVs were assigned to the Bacteroidetes and 519 ASVs to the Proteobacteria, while 985 ASVs remained unassigned or unknown (i.e. NA, table S2). Overall, the most abundant bacteria were *Lacibacter cauensis* (Bacteroidetes, Sphingobacteriia) represented by 43 ASVs, *Devosia* sp (Proteobacteria, α -Proteobacteria) represented by 20 ASVs, *Serratia marcescens* and *Acinetobacter guillouiae* (Proteobacteria, γ -Proteobacteria) represented by 4 and 8 ASVs respectively, as well as *Pseudomonas fluorescens*, *P. unclassified* and *P. vranovensis* (Proteobacteria, γ -Proteobacteria) represented by 51, 11 and 4 ASVs respectively.

Among assigned taxa, bacteria identified to the species level differently composed communities associated to each insect development stage: *A. guillouiae* was more associated to female and male adults G; *S. marcescens*, *A. guillouiae* were more associated to unsterilized eggs G+1 and *Bacillus thuringiensis* was specific to this stage; *L. cauensis* was more associated to sterilized eggs G+1; *L. cauensis* and *Pseudomonas* sp (unknown) were more associated to fed larvae G+1; *L. cauensis*, *S. marcescens*, *A. guillouiae*, *P. fluorescens* and *P. vranovensis* were more associated to starved larvae G+1; *Achromobacter piechaudii*, *Pseudomonas* sp (unknown and unclassified) and *P. vranovensis* were more associated to female empty pupae G+1; *L. cauensis*, *Devosia* sp (unknown), *P. fluorescens*, *Pseudomonas* sp (unclassified) and

P. vranovensis were more associated to male empty pupae G+1; *Erwinia* sp (unknown) and *P. fluorescens* were more associated to unsterilized eggs G+2 and *L. cauensis* was more associated to sterilized eggs G+2 (table S2).

3.2. Vertical microbial transmission

We observed that *S. marcescens* was the only bacterial species 100% shared by all samples of female adults G and unsterilized eggs G+1, but not shared by sterilized eggs G+1 (figure 4). *Bacillus thuringiensis* and *A. guillouiae* were present only in all samples of unsterilized eggs G+1. While the Enterobacteriaceae family was present in all samples of adults G and unsterilized eggs G+1, it was also the only taxon shared by all samples of both male and female adults G. Apart from the order of Pseudomonadales present in all samples of both unsterilized eggs and fed larvae G+1, there was no shared bacterial species between the egg and larval stages (figure S1A). *Pseudomonas* was the only genus shared by all samples of fed larvae and female empty pupae G+1 (figure S1B).

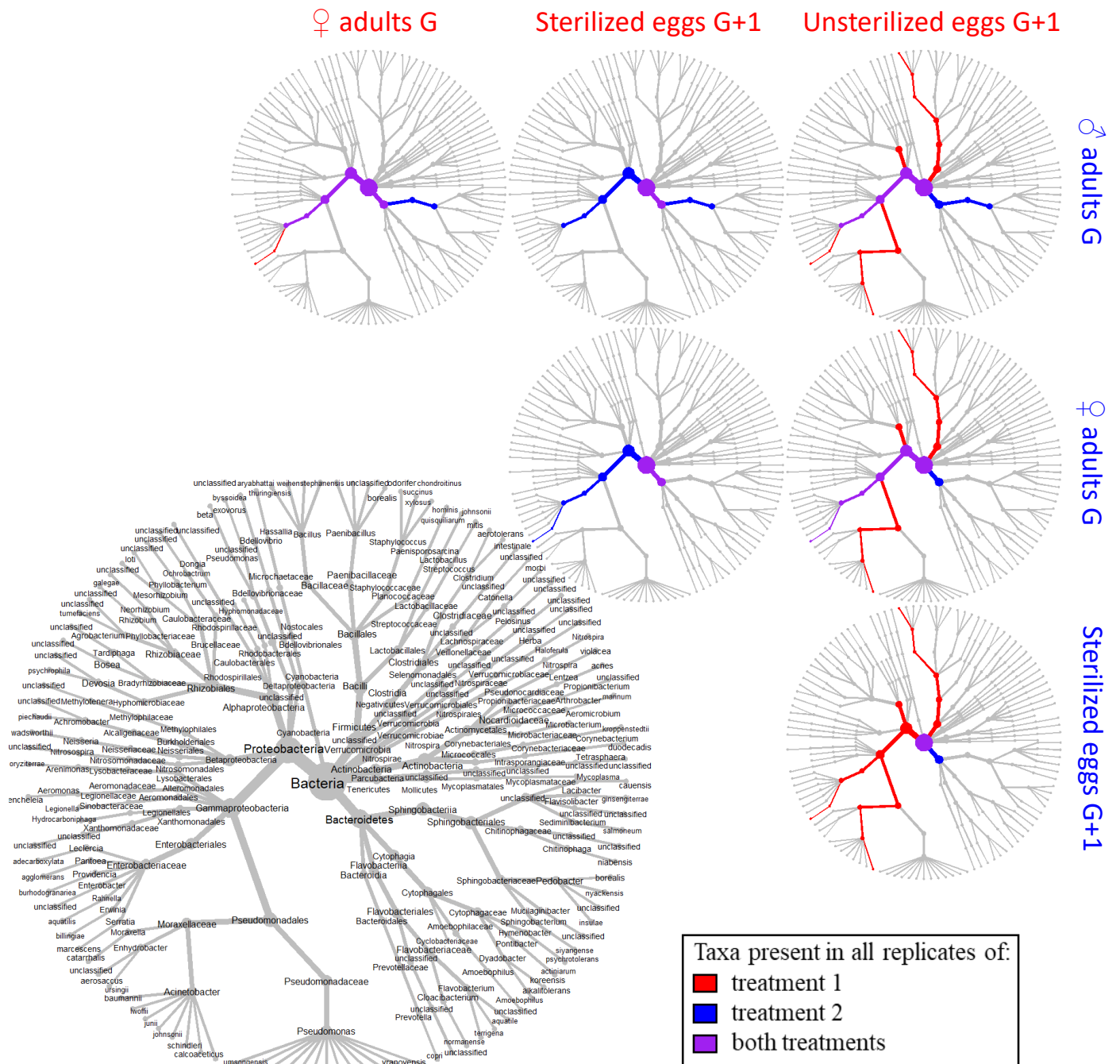


Figure 4 Heat trees comparing taxa between the adult and egg development stages of the CRF. The color of each taxon represents the taxa presence in all replicates of a given treatment. The grey tree on the lower left functions as a key for the smaller unlabeled trees.

Similarly, the two generations of eggs were quite different as only the Pseudomonadales order was shared by all samples of both unsterilized eggs G+1 and G+2 (figure 5). While there was no taxon present specifically in all samples of sterilized eggs from either generation and none shared, *B. thuringiensis*, *A. guillouiae* and *S. marcescens* seemed to be specifically present

in all samples of unsterilized eggs G+1 and *P. fluorescens* in all samples of unsterilized eggs G+2.

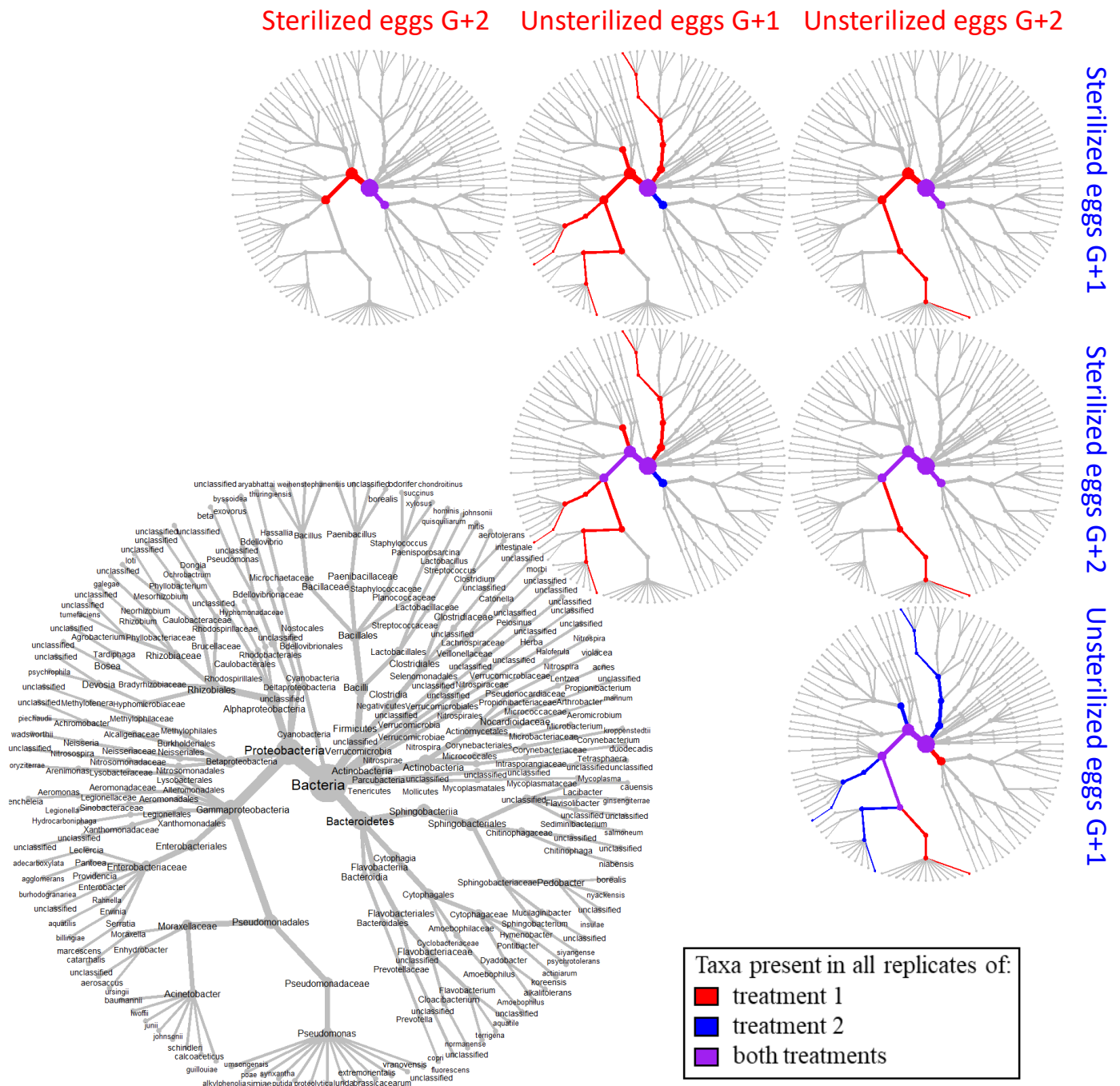


Figure 5 Heat trees comparing taxa between the two generations of eggs of the CRF. The color of each taxon represents the taxa presence in all replicates of a given treatment. The grey tree on the lower left functions as a key for the smaller unlabeled trees.

The previous analyses showed that several bacterial species stood out by either being present in a specific or several development stages of *D. radicum*. Therefore, we tested whether the presence of these targeted species varied across the development of the CRF (table 1). Among the 16 bacterial species that we retained from the previous analyses and already

described in the literature on *D. radicum* (Lukwinski *et al.*, 2006; Bili *et al.*, 2016; Welte *et al.*, 2016), only *L. cauensis* and *Erwinia billingiae* were not influenced by the insect development stage. The first one was consistently present in half of the samples of each stage while the second one was detected in one larval sample only. The presence of the other species significantly varied across the insect development, though only two bacterial species displayed significant pairwise comparisons: *S. marcescens* and *P. fluorescens*. *Serratia marcescens* was present in almost all adult samples, in 2/3 of the eggs G+1 and in half of the larval samples while *P. fluorescens* was detected in half of the larval and empty pupal samples and in 2/3 of the eggs G+2 samples compared to the other stages.

Table 1 Frequency and statistical output of species targeted based on our previous analyses and descriptions from the literature, across the different CRF development stages. Bold p values indicate a significant effect, and different letters on grey highlighted lines show significant pairwise comparisons among stages. Unclassified corresponds to reads having similarities in the *gyrB* database but which sequences do not have a name for a given taxonomic level while NA corresponds to reads with no similarities in the database.
 “*”: $0.05 < P < 0.01$; “**”: $0.01 < P < 0.001$; “***”: $P < 0.001$

Phylum - Class	Species	Adults G	Eggs G+1	Larvae G+1	Empty pupae G+1	Eggs G+2	Insect development stage
Bacteroidetes - Sphingobacteriia	<i>Lacibacter cauensis</i>	4/9	7/18	21/44	4/10	5/15	Chisq=1.15; df=4; P=0.886
Firmicutes - Bacilli	<i>Bacillus thuringiensis</i>	0/9 a	11/18 a	0/44 a	0/10 a	0/15 a	Chisq=44.29; df=4; P=0***
Firmicutes - Clostridia	<i>Clostridium</i> NA	0/9 a	1/18 a	12/44 a	0/10 a	1/15 a	Chisq=13.12; df=4; P=0.014*
Proteobacteria - α -Proteobacteria	<i>Devosia</i> NA	1/9	3/18	8/44	4/10	2/15	Chisq=3.19; df=4; P=0.607
	<i>Agrobacterium tumefaciens</i>	2/9 a	5/18 a	10/44 a	6/10 a	0/15 a	Chisq=14.29; df=4; P=0.008**
Proteobacteria - β -Proteobacteria	<i>Achromobacter piechaudii</i>	0/9 a	0/18 a	17/44 a	6/10 a	0/15 a	Chisq=33.55; df=4; P=0***
Proteobacteria - γ -Proteobacteria	<i>Erwinia billingiae</i>	0/9	0/18	1/44	0/10	0/15	Chisq=1.57; df=4; P=0.872
	<i>Erwinia</i> NA	0/9 a	0/18 a	1/44 a	0/10 a	5/15 a	Chisq=16.25; df=4; P=0.005**
	<i>Providencia burhodogranariae</i>	6/9 a	0/18 a	0/44 a	0/10 a	0/15 a	Chisq=33.43; df=4; P=0***
	<i>Serratia marcescens</i>	8/9 b	10/18 ab	24/44 ab	1/10 a	0/15 ab	Chisq=33.9; df=4; P=0***
	<i>Acinetobacter guillouiae</i>	5/9 a	10/18 a	11/44 a	1/10 a	0/15 a	Chisq=20.99; df=4; P=0***
	<i>Pseudomonas fluorescens</i>	1/9 ab	2/18 a	21/44 b	6/10 b	10/15 b	Chisq=18.11; df=4; P=0.002**
	<i>Pseudomonas</i> NA	0/9 a	1/18 a	17/44 a	6/10 a	1/15 a	Chisq=22.87; df=4; P=0***
	<i>Pseudomonas unclassified</i>	0/9 a	0/18 a	24/44 a	6/10 a	1/15 a	Chisq=39.34; df=4; P=0***
	<i>Pseudomonas vranovensis</i>	0/9 a	0/18 a	21/44 a	7/10 a	0/15 a	Chisq=42.77; df=4; P=0***

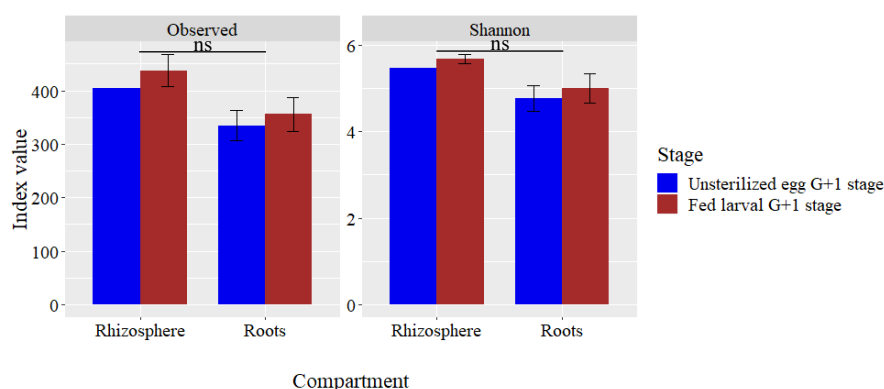
3.3. Horizontal microbial transmission

In order to determine whether the host plant could be involved in microbiota horizontal transmission toward the CRF, the microbiota both from the rhizosphere and the root compartments were first characterized.

After the rarefying step, a total of 25,000 and 150,000 reads was detected in rhizosphere samples collected at the eggs and larval stages respectively, while 150,000 and 175,000 reads were found in root samples, also at egg and larval stages.

For alpha diversity, the number of observed ASVs was not impacted by the plant compartment ($F=4.546$; $df=1$; $P=0.058$) nor by the sampling performed at different insect development stages ($F=0.393$; $df=1$; $P=0.539$, figure 6A). While there were 404 to 437 ASVs in the rhizosphere, a slightly lower number was found for the roots (335 to 355 ASVs). Similarly, the Shannon index was not influenced by the plant compartment ($F=4.131$; $df=1$; $P=0.058$) nor by the sampling performed at different insect development stages ($F=0.429$; $df=1$; $P=0.539$, figure 6A). Again, the rhizosphere tended to be slightly more diverse (5.4-5.6) than roots (4.7-4.9), though the difference was not significant.

A



B

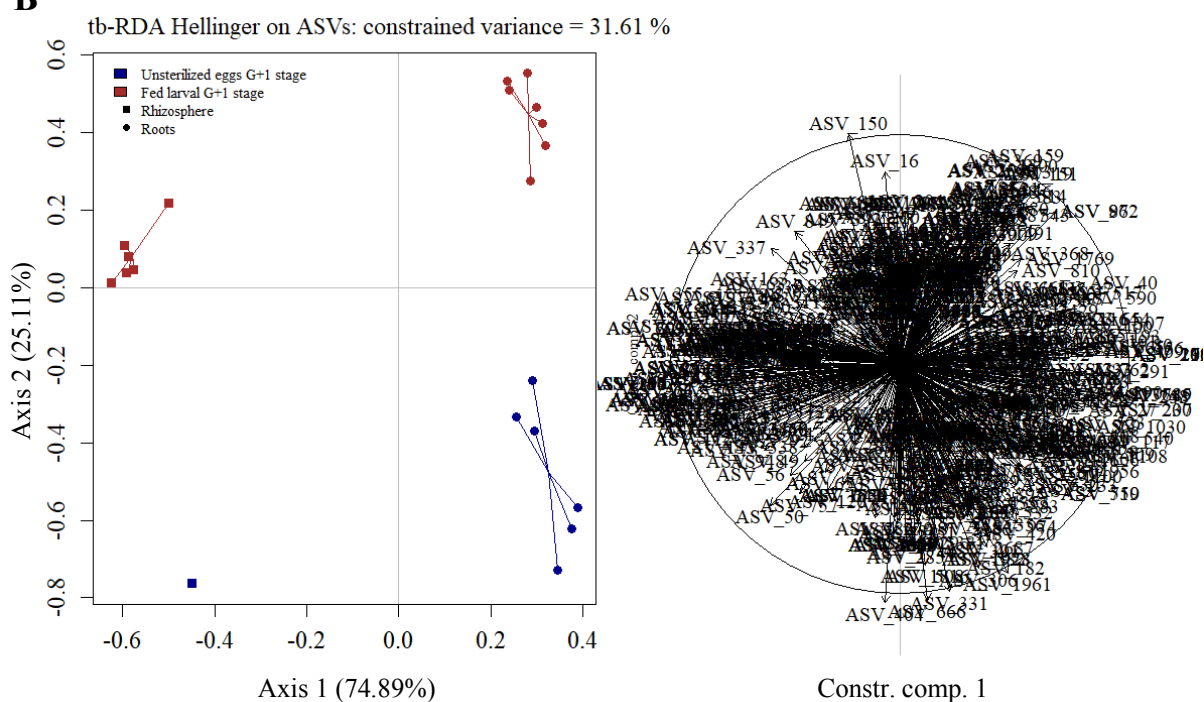


Figure 6 Bacterial diversity of the plant rhizosphere and roots sampled at different insect development stages. A) Alpha diversity is represented by the number of observed ASVs and the Shannon index (mean \pm se). B) Community structure is represented by beta diversity analyzed using a tb-RDA performed on Hellinger-transformed presence/absence data, with sample projection on the left plot and the correlation circle of ASVs on the right one. The constrained variances explained by the axes are given in parenthesis.

Beta diversity, or bacterial community structure was significantly affected by both plant compartments ($F=5.314$; $df=1$; $P=0.001$) and insect development stages ($F=1.991$; $df=1$; $P=0.019$, figure 6B). Our model explained 31.63% of the total constrained variance with the plant compartments accounting for 12.8% and insect development stages for 4.82%.

The most dominant phyla were the Bacteroidetes, Proteobacteria and Firmicutes, with Sphingobacteriia, α - and γ -Proteobacteria as the most abundant classes in the rhizosphere and the roots (figure 7). The minor phyla were the Actinobacteria, Cyanobacteria, Nitrospirae, Parcubacteria, Verrucomicrobia, as well as unknown (i.e. NA) and unclassified (table S3).

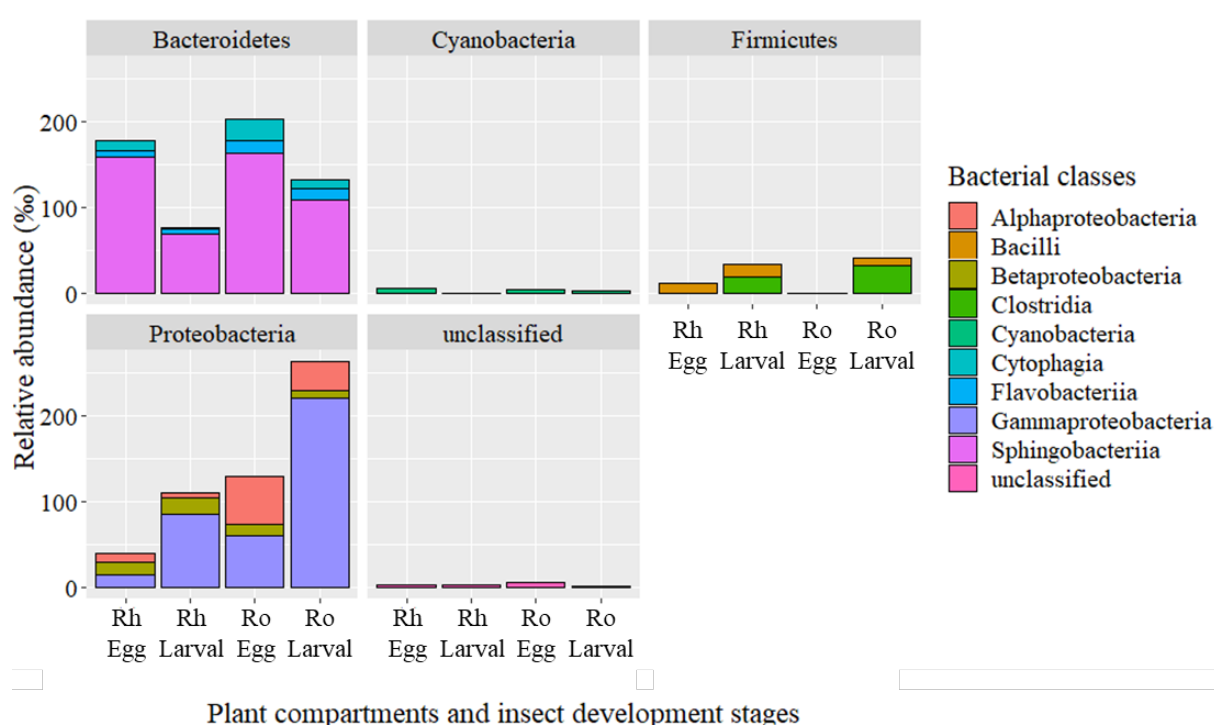


Figure 7 Bacterial phyla and classes of the plant rhizosphere (Rh) and roots (Ro) sampled at different insect development stages: eggs and larvae.

A total of 881 ASVs were detected in rhizosphere and root samples (table S4), with 286 ASVs belonging to the phylum of Bacteroidetes, 242 to Proteobacteria, 63 to Firmicutes, and 261 and 3 respectively to unknown and unclassified phyla (table 3). Regarding assigned taxonomy at the species level, *L. cauensis* (25 ASVs) was the only taxon detected in both rhizosphere and roots at both sampling insect development (egg and larval) stages; *Devosia* sp (12 ASVs) was present in the roots at the egg stage; *Clostridium* sp (23 ASVs), *P. fluorescens* (6 ASVs) and *Pseudomonas* sp (6 ASVs) were present in the roots at the larval stage.

As we wanted to determine whether microbial horizontal transmission occurred when the CRF interacts with its host plant, we compared larval stage microbiota to rhizosphere and root microbiota (figure 8).

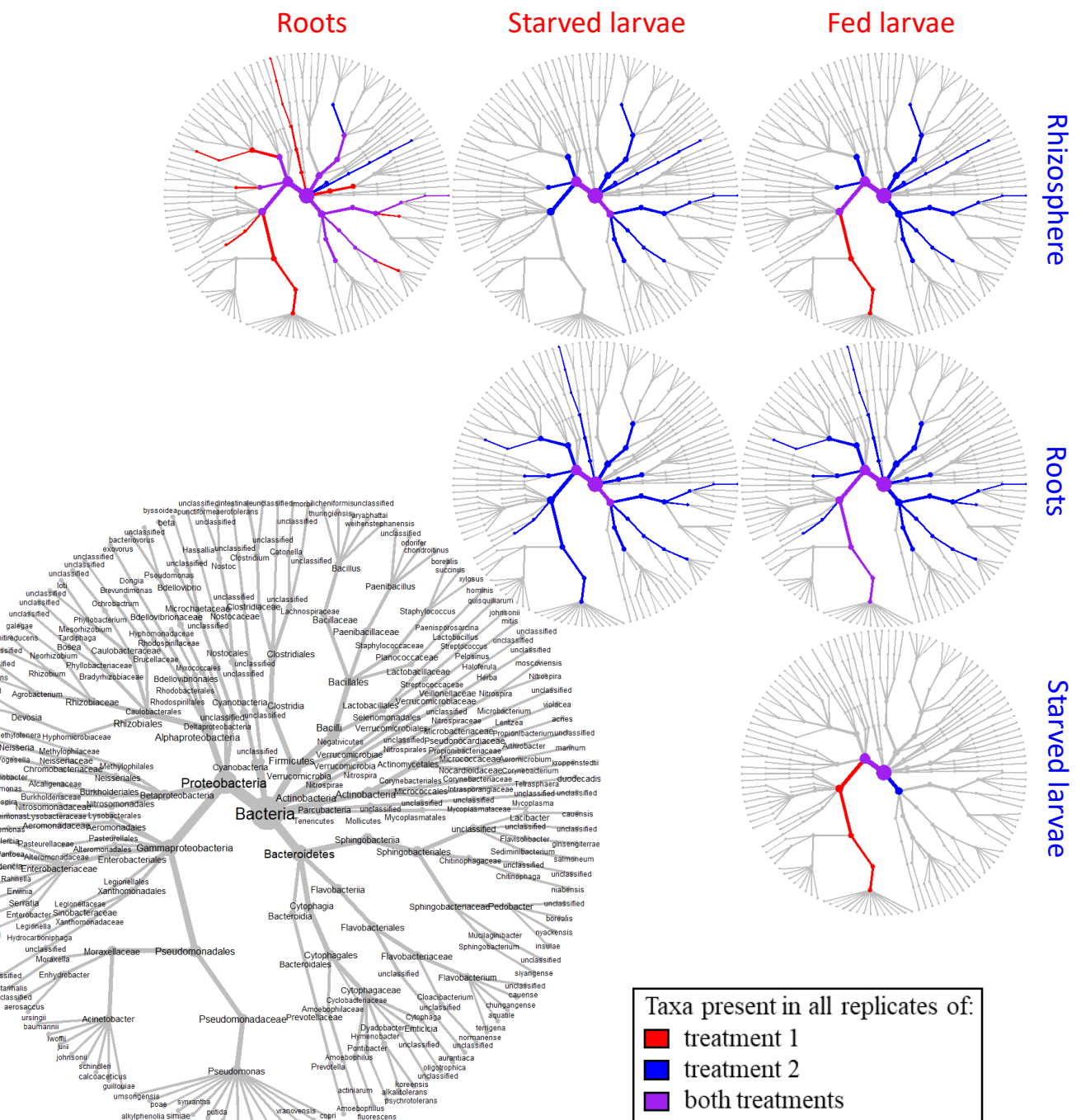


Figure 8 Heat trees comparing taxa between insects, roots and rhizosphere during *D. radicum* larval stage. The color of each taxon represents the taxa presence in all replicates of a given treatment. The grey tree on the lower left functions as a key for the smaller unlabeled trees.

Interestingly, fed larvae shared *Pseudomonas*, as this genus was present in all larval samples, with all root samples, while no bacteria was 100% shared between fed larvae and

rhizosphere samples. Moreover, this genus was not found to be present in all samples of starved larvae even though *P. fluorescens* had a relative abundance of 81‰ in starved larvae and of 16‰ in fed ones (table S2). The heat tree also confirmed the presence of *L. cauensis* in all samples of both rhizosphere and roots, which had a relative abundance of 36 and 31‰ in fed and starved larvae (table S2, figure 8). While this species was not present in all larval samples, it was still detected in all *D. radicum* development stages except in female adults and empty pupae (table 1, table S2).

4. Discussion

Our study showed that *D. radicum* bacterial communities were dynamic as their composition changed throughout the insect development and that several bacterial species were potentially vertically and horizontally transmitted as they were shared between several *D. radicum* stages and between the insect and its host plant. In our study, we detected a certain number of taxa, which were non-assigned at different levels of taxonomy in a relatively homogenous way over all bacterial groups. Poirier *et al.* (2018) demonstrated that some bacterial species, in particular from the Firmicutes phylum, can be under-represented in case they harbor sequences of a *gyrB* paralog, *parE*. Using *gyrB* and *parE* database jointly may reduce taxonomic uncertainty when assigning taxonomy.

4.1. Dynamics of *D. radicum* bacterial communities

At the adult stage, we observed among assigned taxa that *A. guillouiae* was more associated to female and male adults. On the one hand, this species was not detected in the study of Bili *et al.* (2016), which adult flies harbored traces of *Gluconacetobacter*, *Asia*, *Enterococcus*, *Bacillus*, *Deftia*, *Ralstonia* and *Spiroplasma*, but a high abundance of *Wolbachia*. On the other hand, the *Acinetobacter* genus was also observed in a *Wolbachia*-free population, with *Pseudomonas*, *Providencia*, *Serratia* and *Acinetobacter* being the most abundant genera in adult flies (Ourry *et al.* unpublished). The previously mentioned genera are commonly found in other insect species: the burying beetle gut, mainly colonized by γ -Proteobacteria, among which were *Serratia*, *Providencia*, *Erwinia*, *Pseudomonas* and *Acinetobacter* (Heise *et al.*, 2019); the core gut microbiome of coffee berry borer, containing *Pseudomonas*, *Acinetobacter* and *Erwinia* (Ceja-Navarro *et al.*, 2015); the gut of

Lepidopterans, composed of Enterobacteriaceae, Bacillaceae, Pseudomonadaceae (Paniagua Voirol *et al.*, 2018).

We detected bacteria in unsterilized eggs but also in sterilized eggs and bleached-sterilization did affect beta diversity. This suggests that bleaching did remove bacteria that were coating the external surface of the eggs and/or that bacteria may potentially live inside the eggs. However, the extent of bleaching and whether it removed all or part of the external bacteria was not assessed. We also observed that alpha diversity associated to the egg stages did not differ from the other development stages. This seemed to differ from other dipterans. Indeed, Wong *et al.* (2011) found that the sterilized eggs of *Drosophila melanogaster* hosted the most diverse bacterial communities compared to the other development stages. We identified different bacterial species in eggs such as *S. marcescens*, *A. guillouiae*, *Erwinia* sp, *P. fluorescens* belonging to the Proteobacteria; *L. cauensis* belonging to the Bacteroidetes and *B. thuringiensis* belonging to the Firmicutes. Using a cultivable approach, Lukwinski *et al.* (2006) also found diverse bacteria in *D. radicum* eggs, as up to 22 different types of microorganisms were observed from eggs.

In our study, to assess microbiota at the larval stage, a stage that strongly interacts with the host plant, we submitted larvae to starvation during 24 hours to eliminate both root tissues and root-associated microbiota from the larval gut. Thus, we compared starved and fed larvae and observed that starvation did reduce alpha diversity but not beta diversity, which means that communities were less diverse but still had the same structure. Thus, it raises the questions as to whether 24 hours starvation was enough to eliminate root microbiota from the larva gut. In any case, the bacterial communities that we observed came from the overall larvae, including the gut. Still, we identified *L. cauensis* and *Pseudomonas* sp in fed larvae; *L. cauensis*, *S. marcescens*, *A. guillouiae*, *P. fluorescens* and *P. vranovensis* in starved larvae. Both the study of Lukwinski *et al.* (2006) and of Welte *et al.* (2016) also detected *S. marcescens* and *Pseudomonas* in *D. radicum* larval gut, as well as *Acinetobacter* and most gut bacteria belonged to γ -Proteobacteria confirming the prevalence of these species.

In our study, various bacterial species dominantly colonized the different insect stages. Insect development has indeed a major impact on the insect microbiota and both Wong *et al.* (2011) and Staudacher *et al.* (2016) also observed that the microbial community composition of the fly *D. melanogaster* and the moth *Heliothis virescens*, strongly varied between development stages. This variation comes from the underlying consequences of development such as a change of habitat and/or diet. In the case of *D. radicum*, larvae feed on plant roots belowground, pupate in the soil, and emerge as adult flies living aboveground and feeding from

plant nectar. It would not be surprising that such changes have repercussion on the insect symbionts, which must adapt to their new environment and food (Paniagua Voirol *et al.*, 2018).

4.2. Vertical transmission of bacteria

First, we noted that bacterial community structure of *D. radicum* females and eggs were rather similar, which was consistent with the results of Staudacher *et al.* (2016) where a female moth and its eggs tended to cluster together, though the authors did not find strong evidence of bacterial vertical transmission.

Our study showed that *S. marcescens* was shared between all samples of female adults and of unsterilized eggs, as well as *A. guillouiae* though these bacteria were not present in all of these samples. When hatching, *D. melanogaster* larvae eat a part of the egg chorion, which surface is coated with female ovipositor microorganisms, becoming part of larval microbiota (Bakula, 1969). *Delia radicum* first instars are also known to eat their egg shell upon hatching (Johnson, 1930) and they could acquire *S. marcescens* and *A. guillouiae* present on the surface at this moment. Such transmission is plausible as Mariño *et al.* (2018) showed that the *Acinetobacter* genus was shared between the egg and the adult stages of the coffee berry borer and as the study of Sikorowski and Lawrence (1998) demonstrated that *S. marcescens* were vertically transmitted from the female moth to its eggs.

The genera *Serratia* and *Acinetobacter* are also known for having a potential resistance against isothiocyanates, which are toxic plant defenses encountered by developing *D. radicum* larvae (Welte *et al.*, 2016). Ingestion of these bacteria could provide larvae with enough resistance to survive on its host plant upon hatching and continue its development.

We also found that some *Pseudomonas* species were present in both the larval and empty pupal stages but not in the adults from the previous generation. In the literature, *Pseudomonas* is often associated to detoxifying functions. For instance, the *Pseudomonas* present in oral secretion of the Colorado potato beetle larva, was able to suppress the plant defenses during larval feeding (Chung *et al.*, 2013), while *Pseudomonas* from the coffee berry borer gut degraded the coffee plant defenses (Ceja-Navarro *et al.*, 2015). In the case of *D. radicum*, *Pseudomonas* was detected in the larval gut and characterized as being resistant to isothiocyanates, as *Serratia* and *Acinetobacter*, but *Pseudomonas* was classified in the high resistant class (Welte *et al.*, 2016). We could suppose that *S. marcescens* and *A. guillouiae*, potentially transmitted from the females to the eggs, intervene in the early larval instars and contribute to the larval survival by detoxifying the plant defenses, and then *Pseudomonas* could take over their detoxifying functions in the later larval instar. To verify this scenario, both early

and late larval instars should be compared and the functions of their associated microbiota assessed.

The presence of *Pseudomonas* in the empty pupae but not in the adult could be a consequence of metamorphosis that casts aside whatever microorganism is useless to the adult stage. Bakula (1969) observed a potential evacuation of gut microbiota occurring before *D. melanogaster* pupation as the number of bacteria dropped at the beginning of pupation before it regained the same level. We could suggest that pupation functions as a reset button, where the larva gets rid of part of its bacteria, useful at the larval stage to cope with the plant defenses but worthless once at the adult stage. In such a case, either the remaining bacteria refill the ecological niche left emptied after the reset, and/or the adult acquires new bacteria through contamination with its environment. However, such hypotheses are merely speculations as adults associated to their empty pupae were missing in our experiment since they did not have sufficient reads to successfully pass the bioinformatical and cleaning steps. In order to ascertain this hypothesis, comparisons should be made between the larva, pupa and newly-emerged adult stages and assess whether some bacteria were eliminated while others acquired during pupation.

Lastly, we compared the two egg generations in our study to assess vertical transmission of bacteria. We observed differences in alpha and beta diversity with bacterial communities from the second generation being more diverse than the ones from the first generation, and *Pseudomonadales* was the only shared taxa between both generations. These results suggested that there was little to no occurrence of vertical transmission between the two egg generations. In this case, the host plant could play an important part in the acquisition of the microbiota in *D. radicum* as parents of the first egg generation were bred on rutabaga roots, while parents of the second egg generation developed on oilseed rape. Similarly in our previous study, the bacterial community composition of control flies from a *Wolbachia*-free population tended to differ between two generations, which could be due to breeding on different host plants and thus to horizontal transmission (i.e. rutabaga and turnip roots, Ourry *et al.* unpublished).

4.3. Horizontal transmission of bacteria

Pseudomonas was also the only bacterial genus 100% shared by all samples of fed larvae and plant roots and several *Pseudomonas* species were detected in some samples of fed and starved larvae as well as roots. As previously mentioned, *Pseudomonas* has frequently been found to detoxify the plant defenses and promote its insect host development and survival, including in *D. radicum*. Consequently, we could hypothesize that *Pseudomonas* may be acquired by larvae from the roots upon feeding and that the bacteria may provide support to the

larvae to cope with the plant defenses while feeding. Additionally, one study has also reported a case of plant-mediated horizontal transfer involving both *Pseudomonas* and *D. radicum* (Flury *et al.*, 2018). These authors inoculated host plants with a root-colonizing, plant-beneficial and insecticidal bacterium *Pseudomonas protegens* CHA0 and while these bacteria did not affect *D. radicum* survival, their presence was detected in insect larval, pupal and adult stages. Flury *et al.* (2018) also observed that *P. protegens*-infected flies could also transmit the bacteria to a new uninfected host plant. Thus, their study has the particularity to report three types of bacterial transmissions: from the plant to the insect (i.e. horizontal), between insect development stages (i.e. vertical) and from the insect to an uninfected plant (i.e. horizontal).

Lacibacter cauensis was observed in most insect (i.e. eggs, larvae and empty pupae) and plant (i.e. both root and rhizosphere) samples in our study. Not much is known about this bacterium but Qu *et al.* (2009) seemed to be the first to have discovered it in the sediments of a Chinese eutrophic lake. In our previous study, we did not observe the presence of these bacteria in oilseed rape roots and rhizosphere (Ourry *et al.*, 2018), while only traces (< 1%) were detected in the soil by Lachaise *et al.* (2017). In these two studies, the soil matrix used was composed of 1/3 sand and 2/3 soil mixture, gamma-sterilized then inoculated with different microbial diversities that we manipulated. In the present study, we used the same 1/3 sand and 2/3 soil mixture but it was not sterilized in order to identify potential horizontal transmission of bacteria from the soil. We could suppose that sand may be the origin of these bacteria and therefore hypothesize that *L. cauensis* was originally present in the soil at the beginning of the experiment, taken up by the plant and somehow transmitted to the CRF through contact with the plant soil or roots. This hypothesis could be verified by isolating and labelling *L. cauensis* with a fluorescent probe, inoculating the bacteria in the soil during the sowing and evaluating its potential colonization of the plant and the insect with fluorescence microscopy.

5. Conclusion

Our study showed that bacterial composition varied across the development stages of the cabbage root fly and that several bacterial species can be assumed to be vertically transmitted from mothers to eggs (*S. marcescens* and *A. guillouiae*) and potentially horizontally acquired from the plant roots and soil (*Pseudomonas* and *L. cauensis*).

In order to precisely access bacterial transmission in insects, design of specific probe targeting key bacterial species identified in this study could be coupled to fluorescent labelling

to follow microbial transmission between insect stages and generations or from both the host root plant and soil, and relevant insect stages. Although methods are progressing fast for the assessment of such transmissions, the role of insect symbionts in plant-insect interactions is still far from being fully unraveled.

Acknowledgements

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6. Supplemental data

Table S1 Compilation of ASV taxonomic, mean relative abundance ($\% \pm se$) and frequency data for each development stages of *D. radicum*. Unclassified corresponds to reads having similarities in the *gyrB* database but which sequences do not have a name for a given taxonomic level while NA corresponds to reads with no similarities in the database.

<https://drive.google.com/open?id=1k4huhLRvQZElInGskDtsXheavci67t7G>

Table S2 Mean relative abundance (%) of species detected in the different development stages of *D. radicum*. Grey and bold writing identifies the most abundant phyla while double-underlined taxa corresponds to species previously detected in the CRF in according to the literature. Colored figures correspond to abundances superior to 20% for a given development stage and sample characteristic. Unclassified corresponds to reads having similarities in the *gyrB* database but which sequences do not have a name for a given taxonomic level while NA corresponds to reads with no similarities in the database.

Phylum Class	Order	Family	Genus	Species	Number of ASVs	♀ ad. G N=3	♂ ad. G N=6	Unst. eggs G+1 N=10	St. eggs G+1 N=8	Fed L3 G+1 N=26	Starv. L3 G+1 N=18	♀ ep G+1 N=4	♂ ep G+1 N=6	Unst. eggs G+2 N=9	St. eggs G+2 N=6
Actinobacteria					53	18.00	3.33	17.20	16.00	7.73	16.11	8.50	4.83	3.78	0.33
Actinobacteria	Actinomycetales	Microbacteriaceae	<i>Microbacterium</i>	NA	1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.22	0.00
				<i>unclassified</i>	1	0.00	0.00	0.00	0.00	0.00	0.00	7.75	0.00	0.00	0.00
		Micrococaceae	NA	NA	16	17.33	1.00	8.30	2.25	1.15	5.72	0.00	0.00	1.89	0.00
				<i>marinum</i>	1	0.00	0.00	0.00	0.00	0.08	0.00	0.00	0.00	0.00	0.00
		Nocardioidaceae	NA	NA	1	0.00	0.33	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
				<i>acnes</i>	2	0.00	0.00	0.10	0.50	0.35	0.00	0.75	0.17	0.22	0.00
		Propionibacteriaceae	<i>Propionibacterium</i>	<i>violacea</i>	1	0.00	0.00	0.00	3.00	0.00	0.00	0.00	0.00	0.00	0.00
				NA	1	0.00	0.33	0.00	7.25	0.69	0.00	0.00	0.00	1.11	0.00
		Corynebacteriaceae	<i>Corynebacterium</i>	<i>kroppenstedtii</i>	1	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00
				<i>duodecadis</i>	1	0.67	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Micrococcales	Intrasporangiaceae	<i>Tetrasphaera</i>	NA	27	0.00	1.67	8.80	3.00	4.46	10.39	0.00	4.67	0.33	0.33
				NA											
Bacteroidetes					758	178.67	247.00	201.10	288.50	277.46	302.67	179.50	223.50	82.56	292.50
Bacteroidia	Bacteroidales	NA	NA	NA	1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.50
				<i>copri</i>	1	0.00	0.00	0.00	0.00	0.46	0.00	0.00	0.00	0.00	0.00
		Prevotellaceae	<i>Prevotella</i>	<i>Amoebophilus</i>	2	0.00	1.33	0.00	0.00	0.27	0.00	0.00	0.00	0.00	0.17
				NA	2	0.00	0.00	0.00	0.00	0.77	0.50	0.00	0.00	0.00	0.00
		Amoebophilaceae	NA	<i>alkalitolans</i>	1	0.00	0.50	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
				<i>koreensis</i>	2	0.00	0.00	0.00	0.00	0.12	0.00	0.00	0.50	0.00	0.00
		Cytophagaceae	NA	NA	4	0.00	0.17	0.00	0.00	0.50	0.11	0.00	0.00	0.00	0.83
				<i>psychrotolerans</i>	2	0.00	10.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
		Hymenobacter	NA	NA	2	0.00	0.00	2.10	0.00	0.00	0.00	0.00	0.00	0.00	0.00
				<i>actinurum</i>	3	0.00	0.00	0.00	0.00	0.00	5.00	0.00	0.00	3.00	0.00
	Flavobacteriales	Flavobacteriaceae	NA	NA	6	0.00	0.00	2.30	0.00	1.65	0.00	0.00	0.50	0.00	7.17
				<i>normanense</i>	1	0.00	0.00	0.00	0.00	0.12	0.00	0.00	0.00	0.00	0.00
Flavobacteriia	Flavobacteriales	Flavobacterium	<i>Flavobacterium</i>	<i>aquatile</i>	1	0.00	0.00	0.00	0.00	0.23	0.00	0.00	0.00	0.00	0.17
				NA	28	0.00	1.33	6.20	0.00	9.73	17.72	0.00	1.50	1.33	5.50
				<i>terrigena</i>	2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.56	0.00
				<i>unclassified</i>	22	6.67	1.50	0.80	13.63	2.54	32.28	13.00	1.83	4.67	0.83
		NA	NA	NA	20	11.33	0.83	5.30	0.00	4.50	0.44	1.00	0.00	1.11	2.33
				NA	11	0.00	9.33	0.00	1.25	0.96	0.83	0.00	2.33	0.00	0.50
	NA	unclassified	unclassified	<i>unclassified</i>	2	0.00	2.33	0.00	0.00	0.73	0.00	0.00	0.00	0.00	0.00
				NA	1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.67	0.00	0.00
NA	NA	NA	NA	NA	399	122.00	144.50	146.50	187.75	143.92	161.11	69.50	133.17	44.22	170.00
				<i>niabensis</i>	5	0.00	0.00	0.30	0.00	1.23	3.22	4.75	0.00	0.00	0.00
		Chitinophagaceae	NA	NA	7	0.00	0.00	0.20	0.00	6.31	4.83	0.00	0.00	0.00	0.33
				<i>unclassified</i>	6	0.00	0.00	0.00	1.50	1.65	1.28	0.00	0.00	0.00	0.00
		NA	NA	NA	147	38.67	52.50	17.60	56.00	58.92	39.67	71.75	29.67	18.78	67.33
				<i>Mucilaginibacter</i>	1	0.00	0.00	0.00	0.00	0.00	3.39	0.00	0.00	0.00	0.00
	Sphingobacteriales	Sphingobacteriaceae	NA	<i>unclassified</i>	5	0.00	0.33	0.20	0.00	1.54	0.11	0.75	0.00	0.00	1.00
				NA	3	0.00	1.67	0.00	0.00	0.00	0.00	0.00	4.33	0.00	0.50
	NA	NA	<i>Pedobacter</i>	<i>borealis</i>	4	0.00	0.00	0.50	0.00	0.69	0.00	5.75	0.00	0.00	0.00
				<i>insulae</i>	1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	19.67	0.00	0.00

Phylum Class	Order	Family	Genus	Species	Number of ASVs	♀ ad. G N=3	♂ ad. G N=6	Unst. eggs G+1 N=10	St. eggs G+1 N=8	Fed L3 G+1 N=26	Starv. L3 G+1 N=18	♀ ep. G+1 N=4	♂ ep. G+1 N=6	Unst. eggs G+2 N=9	St. eggs G+2 N=6
Sphingobacteria	Sphingobacteriales	Sphingobacteriaceae	<i>Padobacter</i>	NA	2	0.00	0.00	0.00	0.00	0.12	0.00	3.00	0.00	0.00	0.00
				<i>nyackensis</i>	1	0.00	0.00	0.00	0.00	0.15	0.00	0.00	0.67	0.00	0.00
				<i>unclassified</i>	7	0.00	0.00	0.00	0.00	0.19	0.00	8.00	0.83	0.00	2.00
				<i>siyangense</i>	2	0.00	0.00	0.00	0.00	1.46	0.00	0.00	0.00	0.00	0.00
				<i>ginsengiterrae</i>	2	0.00	0.00	0.00	0.00	0.46	0.00	0.00	0.00	0.00	0.00
				<i>cavensis</i>	43	0.00	18.00	19.10	26.88	36.27	31.94	0.00	25.83	8.89	29.50
				NA	7	0.00	2.67	0.00	0.00	1.96	0.22	0.00	0.00	0.00	2.83
				<i>Sediminibacterium</i>	1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	2.00	0.00	0.00
				<i>unclassified</i>	1	0.00	0.00	0.00	0.00	0.00	0.00	2.00	0.00	0.00	0.00
					3	0.00	0.00	1.20	0.00	4.38	0.00	0.00	0.00	0.00	1.33
Cyanobacteria	NA	NA	NA	NA	1	0.00	0.00	0.00	0.00	1.23	0.00	0.00	0.00	0.00	0.00
Cyanobacteria	Nostocales	Microchaetaceae	<i>Hassallia</i>	<i>byssoides</i>	2	0.00	0.00	1.20	0.00	3.15	0.00	0.00	0.00	0.00	1.33
Firmicutes					98	4.33	18.83	177.70	0.13	38.38	8.11	6.25	13.33	4.22	7.50
Bacilli	Bacillales	Bacillaceae	<i>Bacillus</i>	<i>aryabhattai</i>	1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.50
				NA	2	0.00	0.00	0.00	0.00	0.00	7.11	0.00	0.00	0.00	1.33
				<i>thuringiensis</i>	4	0.00	0.00	150.30	0.13	0.00	0.00	0.00	0.00	0.00	0.00
				<i>unclassified</i>	2	0.00	0.00	0.00	0.00	0.35	0.00	0.00	0.00	0.00	0.50
				<i>weihenstephanensis</i>	1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.33
				NA	6	0.00	0.00	2.00	0.00	0.00	0.00	3.25	0.00	0.00	2.50
				NA	7	0.00	4.67	1.80	0.00	1.08	0.00	0.00	0.00	0.00	1.50
				<i>borealis</i>	1	0.00	0.00	0.00	0.00	0.12	0.00	0.00	0.00	0.00	0.00
				<i>chondroitinus</i>	2	0.00	7.83	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
				NA	2	0.00	0.00	0.00	0.00	0.54	0.00	0.00	0.00	0.00	0.00
				<i>odorifer</i>	2	0.00	0.00	0.00	0.00	0.50	0.00	0.00	0.00	0.00	0.00
				<i>unclassified</i>	2	0.00	0.00	0.00	0.00	0.85	0.00	0.00	0.00	0.00	0.00
				<i>paenisporosarcina</i>	1	0.00	0.00	0.00	0.00	0.08	0.00	0.00	0.00	0.00	0.00
				<i>Staphylococcus</i>	1	0.00	0.00	0.00	0.00	0.08	0.00	0.00	0.00	0.00	0.00
				<i>succinus</i>	1	1.33	3.00	0.10	0.00	0.00	0.00	0.00	0.00	0.00	0.00
				<i>xylosus</i>	1	0.00	0.00	0.00	0.00	0.58	0.00	0.00	0.00	0.00	0.00
				<i>johnsonii</i>	1	0.00	0.00	0.00	0.00	0.00	0.11	0.00	0.00	0.00	0.00
				NA	3	0.00	0.00	1.00	0.00	0.00	0.22	0.00	0.00	0.00	0.00
				<i>Streptococcus</i>	1	0.00	0.00	0.00	0.00	0.15	0.00	0.00	0.00	0.00	0.00
				NA	1	0.00	0.00	0.00	0.00	0.08	0.00	0.00	0.00	0.00	0.00
Clostridia	Clostridiales	Clostridiaceae	<i>Clostridium</i>	<i>aerotolerans</i>	5	0.00	0.00	0.00	0.00	3.50	0.00	0.00	0.00	0.00	0.00
				<i>intestinale</i>	1	0.00	0.00	0.00	0.00	0.08	0.00	0.00	0.00	0.00	0.00
				NA	18	0.00	0.00	0.80	0.00	24.15	0.00	0.00	0.00	0.44	0.00
				NA	2	0.00	0.00	2.60	0.00	0.35	0.00	0.00	0.00	0.00	0.00
				<i>morbi</i>	1	0.00	0.00	0.00	0.00	0.08	0.17	0.00	0.00	0.00	0.00
				<i>unclassified</i>	2	0.00	0.00	0.00	0.00	0.12	0.11	0.00	0.00	0.00	0.00
				NA	3	0.00	0.00	0.00	0.00	0.50	0.00	0.00	0.00	0.00	0.00
				<i>unclassified</i>	3	0.00	0.00	0.00	0.00	0.65	0.00	0.00	0.00	0.00	0.00
				NA	1	0.00	0.00	0.00	0.00	0.15	0.00	0.00	0.00	0.00	0.00
				NA	16	3.00	3.33	19.10	0.00	1.81	0.39	3.00	13.33	3.78	0.83
Negativicutes	Selenomonadales	Veillonellaceae	NA	NA	2	0.00	0.00	0.00	0.00	2.27	0.00	0.00	0.00	0.00	0.00
				<i>unclassified</i>	2	0.00	0.00	0.00	0.00	0.35	0.00	0.00	0.00	0.00	0.00
NA					985	286.33	513.00	417.40	585.13	397.62	323.28	409.00	436.50	195.11	480.83
NA	NA	NA	NA	NA	985	286.33	513.00	417.40	585.13	397.62	323.28	409.00	436.50	195.11	480.83

Phylum Class	Order	Family	Genus	Species	Number of ASVs	♀ ad. G N=3	♂ ad. G N=6	Unst. eggs G+1 N=10	St. eggs G+1 N=8	Fed L3 G+1 N=26	Starv. L3 G+1 N=18	♀ e.p. G+1 N=4	♂ e.p. G+1 N=6	Unst. eggs G+2 N=9	St. eggs G+2 N=6
Nitrospirae Nitrospira	Nitrospirales	Nitrospiraceae	Nitrospira	NA	3	0.00	3.67	0.00	10.25	0.00	0.00	0.00	0.00	0.00	0.67
					1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.33	
Parcubacteria unclassified	unclassified	unclassified	unclassified	NA	6	0.00	4.33	0.00	0.00	1.04	0.72	0.00	0.00	0.89	0.00
					5	0.00	4.33	0.00	0.00	0.73	0.72	0.00	0.00	0.89	0.00
Proteobacteria α-Proteobacteria	Caulobacterales NA	Caulobacteraceae NA	NA	NA	519	510.33	147.33	172.90	80.38	260.54	318.61	385.00	285.17	681.67	180.50
					1	0.00	0.00	0.00	0.00	0.00	3.00	0.00	0.00	0.00	0.00
	Rhizobiales	Bradyrhizobiaceae	NA	NA	27	0.00	0.00	18.50	8.25	6.65	6.94	0.00	11.00	0.44	4.17
					3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.33
			Bosea NA	NA	1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.22	0.00
					1	0.00	0.00	0.00	0.00	0.00	4.28	0.00	0.00	0.00	0.00
		Brucellaceae Hyphomicrobiaceae	Tardiphaga Ochrobactrum Devosia	unclassified unclassified NA	2	0.00	0.00	0.00	0.00	0.27	0.00	0.75	0.00	0.00	0.00
					20	17.33	0.00	5.00	11.75	9.96	7.22	0.00	0.00	0.44	0.67
			NA	psychrophila unclassified	8	0.00	0.00	0.00	0.00	9.62	11.56	0.00	1.83	0.00	0.00
					1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	3.33	0.00
		NA	NA	NA	25	20.00	24.33	3.30	1.75	7.81	11.89	22.00	0.00	4.11	1.33
					1	0.00	0.00	0.00	0.00	0.00	0.44	0.00	0.00	0.00	0.00
		Phyllobacteriaceae	Mesorhizobium	unclassified	3	0.00	3.83	0.00	0.00	0.62	0.00	0.00	0.00	10.33	0.00
					3	0.00	0.00	0.00	0.00	0.92	0.00	0.00	0.00	0.00	0.00
		Rhizobiaceae	Phyllobacterium Agrobacterium	unclassified unclassified	1	0.00	0.00	0.00	0.00	0.00	0.28	0.00	0.00	0.00	0.00
					4	0.00	0.83	2.60	0.00	5.38	0.00	16.00	5.50	0.00	0.00
			NA	NA	1	0.00	0.00	2.20	0.00	0.00	0.00	0.00	0.00	0.00	0.00
					3	0.00	2.50	0.50	0.00	0.12	1.17	0.00	0.00	0.00	0.00
		Neorhizobium Rhizobium	unclassified	galegae NA	1	0.00	0.00	0.00	0.00	0.00	0.00	1.25	0.00	0.00	0.00
					4	4.67	0.00	0.00	0.00	0.77	0.17	0.00	0.00	0.00	0.00
	Rhodobacteriales Rhodospirillales	Hyphomonadaceae Rhodospirillaceae	NA	unclassified	6	0.00	0.00	0.00	0.88	0.62	0.00	9.50	5.67	0.00	0.50
					1	0.00	0.00	1.20	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Burkholderiales	Alcaligenaceae	Dongia	unclassified	1	0.00	0.67	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
					10	0.00	0.00	0.00	0.00	8.15	1.61	50.50	5.00	0.00	0.00
			Achromobacter	piechaudii unclassified	2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
					7	23.33	0.00	0.00	0.00	6.54	0.61	0.00	0.00	0.00	0.00
	Methylophilales	Methylophilaceae	NA	NA	1	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00
					1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.33
	NA	NA	NA	unclassified	12	1.67	0.00	7.80	0.00	2.12	0.44	3.00	0.00	0.22	2.67
					1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.67	0.00
	Neisseriales	Neisseriaceae	NA	NA	1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
					1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	14.22	0.00
	Nitrosomonadales	Nitrosomonadaceae	Neisseria	wadsworthii	3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	5.44	40.33
					4	0.00	0.00	0.00	0.00	1.69	0.00	0.00	0.00	0.00	5.83
			Nitrospira	unclassified	2	0.00	17.33	0.00	0.00	0.00	0.00	5.25	12.50	0.00	0.50
					2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.17	0.44	0.50
	Bdellovibrionales Aeromonadales	Bdellovibrionaceae Aeromonadaceae	Bdellovibrio Aeromonas	exovorus encheleia	2	0.00	0.00	0.00	0.00	1.35	0.00	0.00	0.00	0.00	0.00
					1	0.00	0.00	0.00	0.00	1.15	0.00	0.00	0.00	0.00	0.00
	Alteromonadales Enterobacteriales	NA Enterobacteriaceae	NA Enterobacter	NA unclassified	1	0.00	0.00	0.00	0.00	0.08	0.00	0.00	0.00	0.00	0.00
					1	0.00	0.00	0.00	0.00	0.96	0.33	0.00	0.00	0.00	0.00
			Erwinia	unclassified bilingiae	5	0.00	0.00	0.00	0.00	0.15	0.00	0.00	0.00	0.00	0.00
					1	0.00	0.00	0.00	0.00	0.04	0.00	0.00	0.00	66.22	0.00
			Lectercia	adecarboxylata	1	0.67	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
					2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	2.22	0.00
			Pantoea	agglomerans	2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
					2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
					2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
					2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
					2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
					2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
					2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
					2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
					2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
					2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
					2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
					2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
					2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
					2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
					2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
					2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
					2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
					2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
					2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
					2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
					2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
					2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
					2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
					2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
					2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
					2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
					2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
					2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
					2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
					2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
					2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
					2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
					2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
					2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
					2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
					2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
					2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
					2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
					2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
					2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
					2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
					2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
					2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
					2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
					2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
					2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
					2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
					2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
					2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
					2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
					2	0.00									

Phylum Class	Order	Family	Genus	Species	Number of ASVs	♀ ad. G N=3	♂ ad. G N=6	Unst. eggs G+1 N=10	St. eggs G+1 N=8	Fed L3 G+1 N=26	Starv. L3 G+1 N=18	♀ e.p. G+1 N=4	♂ e.p. G+1 N=6	Unst. eggs G+2 N=9	St. eggs G+2 N=6
γ-Proteobacteria	Enterobacteriales	Enterobacteriaceae	<u>Providencia</u>	<i>burhodograriaea</i>	1	5.00	5.33	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
			<i>Rahnella</i>	<i>aquatilis</i>	2	0.00	8.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Legionellales	Legionellaceae	<i>Serratia</i>	<i>marcescens</i>	4	17.00	6.00	32.80	0.00	17.42	45.78	0.00	0.17	0.00	0.00
			<i>Legionella</i>	NA	2	0.00	0.00	0.00	0.00	1.12	0.00	0.00	0.00	0.00	0.00
	Lysobacteriales	Lysobacteraceae	<i>Arenimonas</i>	<i>oryziterrae</i>	1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
			NA	NA	116	66.67	23.33	47.40	36.25	62.04	20.28	9.50	34.00	24.00	95.83
	Pseudomonadales	Moraxellaceae	<u>Acinetobacter</u>	<i>baumannii</i>	1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.44	0.00
				<i>calcoaceticus</i>	1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	10.50	0.00	0.00
	Pseudomonadaceae			<i>guillouiae</i>	8	251.00	31.83	30.50	0.00	1.92	44.44	0.00	7.00	0.00	0.00
				<i>Johnsonii</i>	2	0.67	0.00	0.90	0.00	0.42	0.00	0.00	0.00	0.00	0.00
				<i>junii</i>	1	0.00	0.00	0.00	0.00	0.00	0.33	0.00	0.00	0.00	0.00
				<i>Iwoffii</i>	1	0.00	0.00	0.00	0.00	0.15	0.00	0.00	0.00	0.11	0.00
				<i>schindleri</i>	1	0.00	0.00	0.00	0.00	0.00	2.89	0.00	0.00	0.78	0.00
				<i>ursingii</i>	1	0.00	0.00	0.00	0.00	0.08	0.00	0.00	0.00	0.00	0.00
			<i>Enhydrobacter</i>	<i>aerosaccus</i>	2	0.00	0.00	0.00	0.00	1.00	0.50	0.00	0.00	0.00	0.00
				<i>unclassified</i>	5	0.00	0.00	0.00	0.00	0.92	0.00	0.00	0.00	0.00	3.17
			<i>Moraxella</i>	<i>catarrhalis</i>	1	0.00	0.00	0.00	0.00	0.08	0.00	0.00	0.00	0.00	0.00
				<i>alkyphenolia</i>	1	0.00	0.00	0.00	0.00	1.12	0.06	0.00	0.00	0.00	0.00
			<u>Pseudomonas</u>	<i>brassicacearum</i>	1	0.00	0.00	0.00	0.25	1.19	1.61	11.00	2.67	0.00	0.00
				<i>extremorientalis</i>	3	0.00	0.00	0.00	0.00	9.54	0.00	0.00	0.00	8.67	0.00
				<i>fluorescens</i>	51	0.00	1.17	2.10	0.00	16.85	81.56	7.50	23.17	504.00	0.17
				<i>lurida</i>	2	0.00	5.50	0.00	8.50	0.92	0.00	0.00	0.00	4.78	0.00
				NA	17	0.00	0.00	0.40	0.00	38.42	2.89	26.50	3.50	0.22	0.00
				<i>poae</i>	1	0.00	0.00	0.00	0.00	0.12	0.00	0.00	0.00	0.00	0.00
				<i>proteolytica</i>	1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
				<i>putida</i>	3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	5.11	0.00
				<i>simiae</i>	1	0.00	0.00	0.00	0.00	0.00	0.00	5.75	4.83	2.56	0.00
				<i>syntantha</i>	3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	2.00	0.00
				<i>umsongensis</i>	1	0.00	0.00	0.00	0.00	0.08	0.00	0.00	0.00	5.11	0.00
				<i>unclassified</i>	11	0.00	0.00	0.00	0.00	8.12	12.33	127.25	21.83	0.56	0.00
			<i>Hydrocarboniphaga</i>	<i>vranovensis</i>	4	0.00	0.00	0.00	0.00	8.62	29.33	56.00	71.67	0.00	0.00
				NA	1	1.00	0.00	0.70	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Xanthomonadales	Sinobacteraceae	NA	NA	7	0.00	0.00	1.10	3.88	0.77	0.56	0.00	0.00	0.11	0.00
		Xanthomonadaceae			1	0.00	0.00	0.00	0.00	0.12	0.00	0.00	0.00	0.00	0.00
NA	unclassified	unclassified	NA	NA	81	101.33	16.67	6.30	8.88	24.58	29.11	59.25	28.00	4.11	24.67
			<i>Pseudomonas</i>	<i>unclassified</i>	2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	2.50	10.11	0.00
Tenericutes	Mollicutes	Mycoplasmatales	Mycoplasmataceae	<i>beta</i>	1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	2.17	0.00	0.00
				NA	1	0.00	0.00	0.00	0.25	0.00	0.00	0.00	0.00	0.00	0.00
Unclassified	unclassified	unclassified	unclassified	unclassified	25	0.00	56.33	10.80	10.75	6.27	28.78	9.75	25.83	0.33	19.33
					25	0.00	56.33	10.80	10.75	6.27	28.78	9.75	25.83	0.33	19.33
Verrucomicrobia	NA	NA	NA	NA	15	0.00	1.33	0.00	8.00	0.08	0.00	1.75	8.17	7.22	1.17
					11	0.00	1.33	0.00	0.00	0.00	0.00	0.00	8.17	7.22	0.33
Verrucomicrobia	unclassified	unclassified	<i>Herba</i>	<i>unclassified</i>	2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.83
Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobiaceae	<i>Halofenula</i>	<i>unclassified</i>	1	0.00	0.00	0.00	0.00	0.08	0.00	0.00	0.00	0.00	0.00
			NA	NA	1	0.00	0.00	0.00	0.00	0.00	0.00	1.75	0.00	0.00	0.00

Abbreviations: ♀, female; ♂, male; ad., adults; unst./st. eggs, unsterilized/sterilized eggs; L3., 3rd instar larvae; starv., starved; e.p., empty pupae.

TableS3 Mean relative abundance (%) of species detected in the different plant compartments (rhizosphere and roots) sampled during *D. radicum* egg and larval stages. Grey and bold writing identifies the most abundant phyla. Colored writing corresponds to abundances superior to 20% for given development stage and sample characteristic. Unclassified corresponds to reads having similarities in the gyrB database but which sequences do not have a name for a given taxonomic level while NA corresponds to reads with no similarities in the database.

Phylum Class	Order	Family	Genus	Species	Number of ASVs	Rhizosphere - egg stage N=1	Rhizosphere - larval stage N=6	Roots - egg stage N=6	Roots - larval stage N=7
Actinobacteria					11	0	2.17	1.00	6.43
Actinobacteria	Actinomycetales	Microbacteriaceae	<i>Microbacterium</i>	<i>unclassified</i>	1	0	0.00	0.00	0.29
		NA	NA	NA	2	0	0.17	0.00	0.57
	NA	NA	NA	NA	8	0	2.00	1.00	5.57
Bacteroidetes					286	272	119.50	443.17	249.43
Cytophagia	Cytophagales	Cyclobacteriaceae	NA	NA	1	0	0.00	1.00	0.00
		Cytophagaceae	<i>Cytophaga</i>	<i>aurantiaca</i>	1	9	0.00	10.17	0.00
			<i>Dyadobacter</i>	NA	1	0	0.17	0.00	0.86
			<i>Emticicia</i>	<i>oligotrophica</i>	2	0	0.17	0.17	2.29
			NA	NA	1	0	0.17	0.00	0.29
			<i>unclassified</i>	<i>unclassified</i>	1	0	0.00	0.00	0.43
		NA	NA	NA	10	2	2.00	14.50	7.14
Flavobacteriia	Flavobacteriales	Flavobacteriaceae	<i>Flavobacterium</i>	<i>cauense</i>	2	1	0.00	1.17	0.14
				<i>chungangense</i>	1	2	0.00	1.00	0.00
				NA	5	1	3.50	1.67	4.43
				<i>unclassified</i>	11	0	0.50	6.50	4.14
			NA	NA	6	2	1.83	1.00	2.29
		NA	NA	NA	5	1	0.00	3.50	1.00
NA	NA	NA	NA	NA	148	94	41.17	237.67	115.71
Sphingobacteriia	Sphingobacteriales	Chitinophagaceae	NA	NA	4	0	0.67	4.67	8.57
		NA	NA	NA	51	16	15.67	69.67	54.57
		Sphingobacteriaceae	NA	NA	2	0	0.33	0.83	1.86
			<i>Pedobacter</i>	<i>borealis</i>	1	0	0.17	0.50	0.43
				NA	1	0	0.50	0.00	0.71
				<i>nyackensis</i>	2	0	1.17	0.17	0.71
				<i>unclassified</i>	1	0	0.00	0.17	1.57
		unclassified	<i>Lacibacter</i>	<i>cauensis</i>	25	143	51.33	86.00	39.86
			NA	NA	3	1	0.17	2.00	2.00
			<i>unclassified</i>	<i>unclassified</i>	1	0	0.00	0.83	0.43
Cyanobacteria					6	6	1.50	5.67	4.86
Cyanobacteria	NA	NA	NA	NA	2	0	0.17	1.67	0.86
	Nostocales	Microchaetaceae	<i>Hassallia</i>	<i>byssioidea</i>	1	6	1.17	3.83	1.29
		Nostocaceae	<i>Nostoc</i>	<i>punctiforme</i>	2	0	0.17	0.17	2.00
				<i>unclassified</i>	1	0	0.00	0.00	0.71
Firmicutes					63	13	42.50	3.17	53.14
Bacilli	Bacillales	Bacillaceae	<i>Bacillus</i>	<i>licheniformis</i>	1	0	0.33	0.00	0.00
				NA	1	0	0.33	0.00	0.00
			NA	NA	9	10	9.33	0.00	0.71
		NA	NA	NA	6	2	3.33	0.17	1.86
		Paenibacillaceae	<i>Paenibacillus</i>	NA	1	0	0.17	0.00	1.43
				<i>odorifer</i>	1	0	0.17	0.00	0.43
				<i>unclassified</i>	3	0	2.00	0.00	4.43
Clostridia	Clostridiales	Clostridiaceae	<i>Clostridium</i>	<i>aerotolerans</i>	1	0	0.33	0.00	0.57
				NA	23	0	17.17	0.00	30.29
			NA	NA	1	0	0.33	0.00	0.00
		NA	NA	NA	3	0	2.67	0.00	2.43
NA	NA	NA	NA	NA	11	1	5.83	3.00	7.43
Negativicutes	Selenomonadales	Veillonellaceae	NA	NA	2	0	0.50	0.00	3.57
NA					261	159	194.17	93.50	94.00
NA	NA	NA	NA	NA	261	159	194.17	93.50	94.00
Nitrospirae					3	1	2.17	0.00	0.14
Nitrospira	Nitrospirales	Nitrospiraceae	<i>Nitrospira</i>	<i>moscoviensis</i>	1	0	0.33	0.00	0.00
				NA	2	1	1.83	0.00	0.14
Parcubacteria					3	3	1.67	1.50	0.71
unclassified	unclassified	unclassified	<i>unclassified</i>	<i>unclassified</i>	3	3	1.67	1.50	0.71
Proteobacteria					242	47	139.50	142.50	292.71
α -Proteobacteria	Caulobacterales	Caulobacteraceae	<i>Brevundimonas</i>	NA	1	0	0.00	0.67	0.00
	NA	NA	NA	NA	16	1	3.83	11.00	14.43
	Rhizobiales	Bradyrhizobiaceae	<i>Bosea</i>	<i>unclassified</i>	3	0	0.00	3.67	0.71
		Hyphomicrobiaceae	<i>Devosia</i>		12	3	1.00	24.50	8.43
				<i>psychrophila</i>	2	2	0.00	2.50	0.00
				<i>unclassified</i>	3	0	0.00	1.83	0.00
			NA	NA	12	5	1.83	10.33	5.29
		Phyllobacteriaceae	<i>Mesorhizobium</i>	<i>unclassified</i>	1	0	0.00	0.67	0.14
	Rhizobiales	Rhizobiaceae	<i>Agrobacterium</i>	<i>tumefaciens</i>	1	0	0.33	0.00	1.86
			<i>Rhizobium</i>		1	0	0.00	0.17	1.29
				<i>selenitireducens</i>	1	0	0.00	0.50	0.00
				<i>unclassified</i>	3	0	0.17	0.50	2.29

Phylum Class	Order	Family	Genus	Species	Number of ASVs	Rhizosphere - egg stage N=1	Rhizosphere - larval stage N=6	Roots - egg stage N=6	Roots - larval stage N=7
β-Proteobacteria	Burkholderiales	Alcaligenaceae	<i>Achromobacter</i>	<i>piechaudii</i>	2	0	1.00	0.00	1.00
		Burkholderiaceae	<i>Chitinimonas</i>	NA	1	0	0.00	9.17	0.00
		NA	NA	NA	1	0	0.17	1.50	1.29
	NA	NA	NA	NA	5	0	2.50	2.00	2.43
	Neisseriales	Chromobacteriaceae	<i>Vogesella</i>	<i>unclassified</i>	1	0	0.17	0.33	0.57
δ-Proteobacteria	Nitrosomonadales	Nitrosomonadaceae	NA	NA	6	14	14.50	1.83	3.86
	Bdellovibrionales	Bdellovibrionaceae	<i>Bdellovibrio</i>	<i>bacteriovorus</i>	1	0	0.33	0.00	0.00
				<i>exovorus</i>	1	0	0.00	0.50	0.14
	Myxococcales	NA	NA	NA	1	0	0.33	0.00	0.00
	NA	NA	NA	NA	1	0	0.33	0.00	0.00
γ-Proteobacteria	Aeromonadales	Aeromonadaceae	<i>Aeromonas</i>	<i>encheleia</i>	1	0	0.33	0.00	0.57
				NA	1	0	0.00	0.00	0.29
	Alteromonadales	Alteromonadaceae	NA	NA	1	0	0.33	0.17	0.00
	Enterobacteriales	Enterobacteriaceae	<i>Enterobacter</i>	<i>unclassified</i>	1	0	0.17	0.00	0.29
	Legionellales	Legionellaceae	<i>Legionella</i>	NA	3	0	0.33	1.67	2.14
				NA	2	0	2.67	0.33	0.14
	NA	NA	NA	NA	90	15	68.83	43.17	85.71
	Pasteurellales	Pasteurellaceae	NA	NA	1	0	0.67	0.00	0.00
	Pseudomonadales	Pseudomonadaceae	<i>Pseudomonas</i>	<i>brassicacearum</i>	3	0	0.50	0.33	15.86
				<i>fluorescens</i>	6	0	3.17	1.17	60.57
				NA	4	0	1.33	0.00	12.00
				<i>stutzeri</i>	1	0	0.00	0.33	0.14
				<i>unclassified</i>	6	0	2.67	0.00	20.86
				<i>vranovensis</i>	2	0	4.67	0.00	11.43
				NA	3	0	1.17	13.67	10.57
NA	NA	NA	NA	NA	41	7	26.17	10.00	28.43
Unclassified	unclassified	unclassified	unclassified	unclassified	3	3	3.00	6.67	1.86
unclassified	unclassified	unclassified	unclassified	unclassified	3	3	3.00	6.67	1.86
Verrucomicrobia	NA	NA	NA	NA	3	1	2.67	0.00	0.14
NA	NA	NA	NA	NA	1	1	1.83	0.00	0.14
Verrucomicrobiae	Verrucomicrobiales	NA	NA	NA	2	0	0.83	0.00	0.00

Table S4 Compilation of ASV taxonomic, mean relative abundance (% ± se) and frequency data for the rhizosphere and root compartments sampled at *D. radicum* egg and larval stages. Unclassified corresponds to reads having similarities in the *gyrB* database but which sequences do not have a name for a given taxonomic level while NA corresponds to reads with no similarities in the database.

<https://drive.google.com/open?id=1Kzdhkx-rXSAM7OvNTmFdkf6hiwdHCHHA>

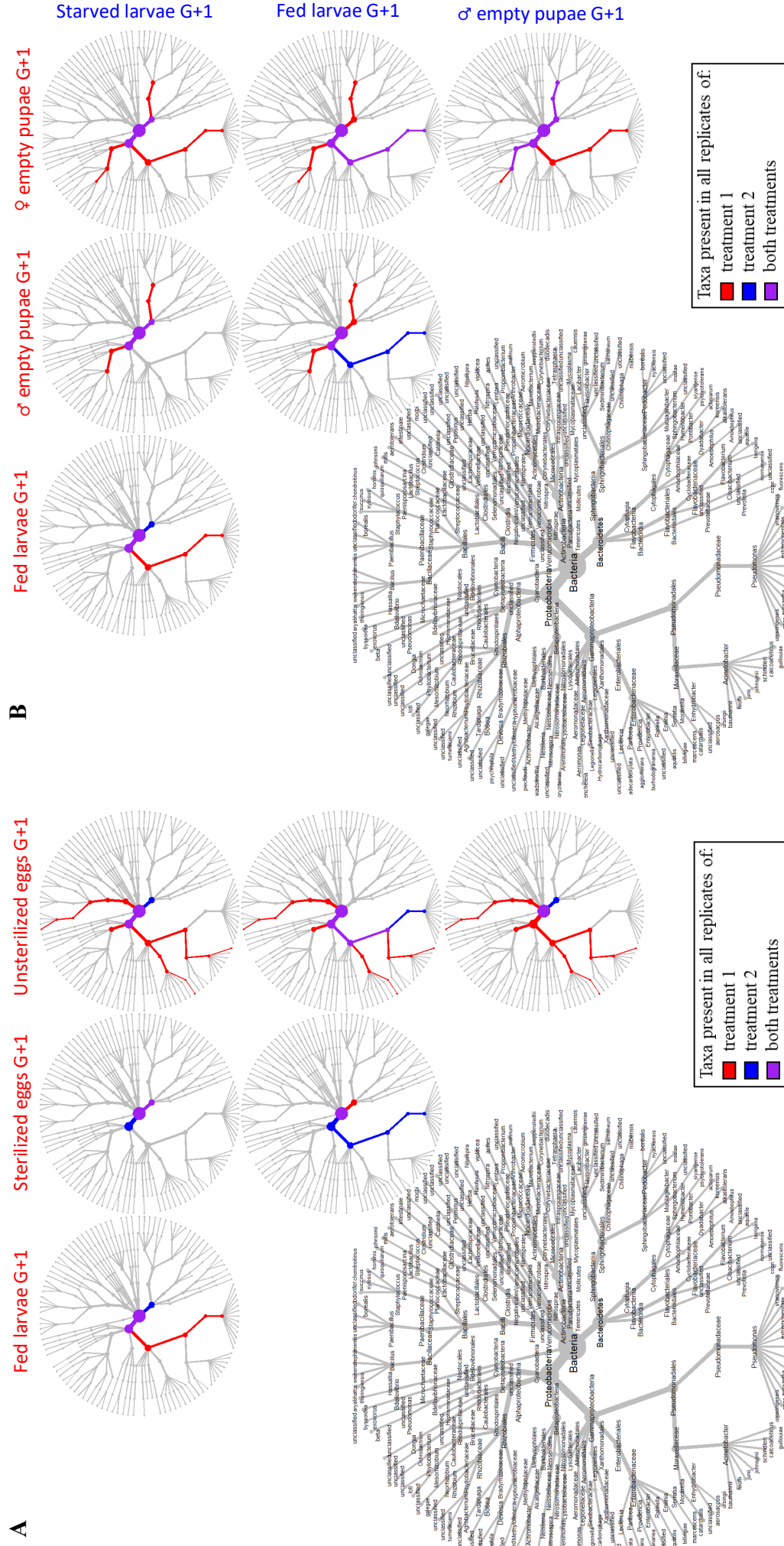


Figure S1 Heat trees comparing taxa between egg and larval stages (A) and between larval and empty pupal stages (B). The color of each taxon represents the taxa presence in all replicates of a given treatment. The grey tree on the lower left functions as a key for the smaller unlabeled trees.

CHAPTER V

GENERAL DISCUSSION AND PERSPECTIVES



CHAPTER V: GENERAL DISCUSSION AND PERSPECTIVES

General discussion

1. Results overview and organization of the discussion

This thesis aimed at understanding the tripartite interaction between the winter oilseed rape (*Brassica napus*), the cabbage root fly (*Delia radicum*) and their associated microbiota (chapter I, figure 13). The first objective was to determine whether a variation of soil microbial diversity could negatively affect *D. radicum* development and behavior through potential modification of plant defenses (chapter II). The second objective was to assess how the insect root herbivory influences back the plant root chemistry and microbial communities throughout the insect development and to identify potential relationships between chemical and microbial modifications following herbivory (chapter III). The third and last objective was to evaluate insect bacterial communities to understand how bacteria respond to antibiotic manipulation and how bacteria are transmitted to their insect host (chapter IV).

Figure 1 summarizes the main results presented in this manuscript.

In the first part of this manuscript (chapter II), we demonstrated that our manipulation of soil microbial diversity through dilution to extinction method, modified *D. radicum* emergence rate (i.e. higher on the medium diversity) and choice oviposition (i.e. higher on low diversity when plants were 4 week-old but on medium diversity when they were 6 weeks-old). However the plant chemical compounds we measured were not influenced by soil microbial diversity, thus we could not link the obtained fly phenotype to the plant chemistry.

In the second part of this manuscript (chapter III), we showed that *D. radicum* herbivory modified root chemistry and microbial communities, especially at the larval stage, and the plant was hypothesized to use its chemistry to attract microorganisms which might promote the plant defenses against the root herbivore.

In the third and last part of this manuscript (chapter IV), we observed on the one hand, that when adult flies were given tetracycline, it modified adult *D. radicum* bacterial communities without suppressing them and that effects of the antibiotic were still visible after several generations. On the other hand, we observed that several bacteria, which potentially could be involved in plant defense detoxification, were shared (and thus potentially transmitted) between *D. radicum* females and eggs and between fed larvae and *B. napus* roots.

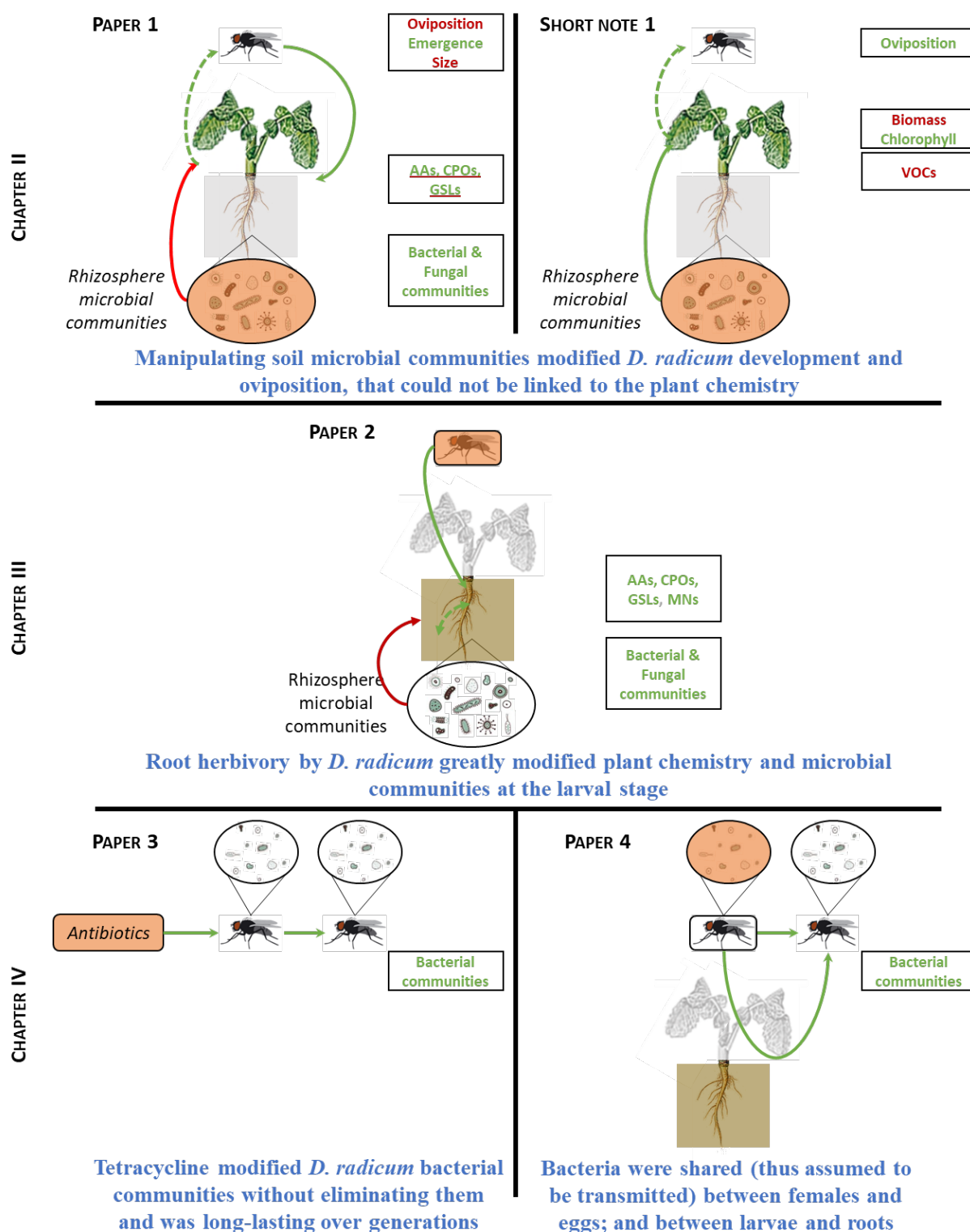


Figure 1 Overview of the main results written in blue detailed in this manuscript. The studied factors are colored in orange while their studied significant and non-significant effects are represented respectively by green and red texts and arrows. Chemical compounds underlined in red colors indicate that they were not impacted by soil microbial variation while dashed arrows indicates effects that we observed but could not explain in our studies. AAs: Amino Acids; CPO: Carbohydrate, Polyol and Organic Acids; GSLs: Glucosinolates; VOCs: Volatile Organic Compounds; MNs: Macro- and micronutrients.

Hereafter the results obtained during this thesis will be discussed, coupled with the limitations of our approaches for each chapter. Improvements and leads will be proposed to fill the gaps left by these limitations. This discussion will be divided into four axes: i) plant chemistry, as a link between soil microorganisms and herbivores, ii) plant chemistry, as a connection between plant and soil microbial communities upon root herbivory, iii) bacterial legacy in insects, and iv) getting closer to functional approaches. Lastly, perspectives will be presented and followed by the conclusion of this work.

2. Plant chemistry, as intermediate between soil microorganisms and herbivore?

In the first part of this manuscript, we showed that both emergence rate and choice oviposition were modulated by soil microbial diversity. We measured several plant chemical traits (i.e. AAs, CPOs, GSLs and VOCs) in order to explain the observed fly phenotype but the plant chemistry was not influenced by soil microbial diversity.

2.1. Spatiotemporal scale and detection resolution to considerate for analysis of plant chemistry

In our study, we found that manipulating soil microbial diversity modified the fly phenotype as in the study of Hol *et al.* (2010) This effect may be important for the fly and its fitness, and thus it may participate to the implementation of plant defenses. Hence, we tried to determine whether this effect on the fly passes through the modulation of plant defenses but ultimately, we did not manage to link the fly phenotype influenced by soil microbial diversity to the chemical compounds we measured.

A first hypothesis would be that microbial diversity was not diluted enough to affect the plant chemistry. Using the same dilution to extinction method to manipulate soil microbial diversity, Hol *et al.* (2010) demonstrated that diverse soil communities were associated to higher total glucosinolate (GSL) and lower total amino acid (AA) contents in presence of a herbivore. In our study, the final content of soil from each dilution or modality (i.e. high, medium and low) was of the same range as in the study of Hol *et al.* (2010), thus excluding the hypothesis of our soil inocula being not diluted enough to influence plant chemistry.

Another hypothesis would be that the chemical compounds we measured did change according to soil microbial diversity but not in the plant compartments that we focused on. We measured total GSLs in leaves and roots, which content was slightly higher in leaves but herbivory decreased GSL contents of both compartments. Interestingly, levels of GSLs can vary between and within plant compartments and the presence of a herbivore differently modulates these levels. For instance, Tsunoda *et al.* (2018) showed that herbivory of coleopteran larvae induced changes in total GSL concentration in tap, lateral and fine roots of *Brassica* species but not in leaves while it slightly changed their contents in petiole and stem of *Sinapis alba* but not in roots. Indole GSLs and more precisely glucobrassicin had varying levels within the different root parts while neoglucobrassicin varied within the different leaf parts following herbivory. Moreover, cotyledons can also contain high levels of GSLs when the plant is at the seedling stage (Hopkins *et al.*, 2009). Li *et al.* (2000) showed that the higher concentration of GSLs in cotyledons, the stronger the decline of the herbivore weight. However, GSL activities decrease as the cotyledons age (Wallace and Eigenbrode, 2002). Plant chemistry is subjected to great spatiotemporal variations: on both large and fine scales (i.e. between and within plant compartments) and during the plant development from seedling to reproductive stage. Our study was performed on *B. napus* vegetative stage using the whole root system and three middle leaves for chemical analyses and the scale of our study may have been too narrow. Further researches would gain to be more integrative and should extent the scale of studies to encompass fine scale analysis and the entire plant biological cycle.

Another hypothesis would be that chemical changes did occur in roots or leaves but were not measured either due to technological limitations or because we chose to focus on certain chemical groups as plant chemistry encompasses a very wide panel of compounds and other groups may have been modulated by soil microbial diversity. In our study, total GSL content, which included five different GSLs was not influenced by the soil microbial diversity. Similarly, Kabouw *et al.* (2011) showed that their soil treatment (i.e. with or without microorganisms, removed by filtering) did not modify total GSL contents from the phloem, leaves and roots. However, Hubbard *et al.*, (2019) using the same filtering method to manipulate the soil microbial communities observed that this treatment influenced only one leaf GSL out of nine measured. However, the GSL family is larger than five or nine compounds: Griffiths *et al.* (2001) detected 28 of them in leaves of 18 wild plant species from the Brassicaceae family and at least 120 different GSLs have been identified in this family according to Hopkins *et al.* (2009). At the time of our study, we were able to properly identify and quantify five GSLs due to the resolution limitation of the instruments and methods we used and it is very likely that we

may have missed some GSLs modulated by soil microorganisms. Since then, a project (“DEsCriBe”) has started in our laboratory, aiming to characterize the diversity of glucosinolates in 304 cultivars of Brassicaceae (see poster of Gravot *et al.* in the chapter “Scientific commitments”). So far, 41 different GSLs have been detected, along with more than 50 phenolic compounds, which have required great methodology, detection and quantification development to reach such results. Lastly, GSLs are only a subgroup within a group of secondary metabolites, which are direct chemical defenses (Chen, 2008; Fürstenberg-Hägg *et al.*, 2013) and we could hypothesize that other groups secondary metabolites, physical or indirect defenses (Aljbory and Chen, 2018) that we did not measured, may have reacted toward soil microbial manipulation.

2.2. Toward genericity of our results

In chapter II, our experiments were performed using the same soil from the same field (i.e. La Gruche, Brittany, France), where conventional agriculture is applied. Upon manipulating this soil microbial diversity used as inoculum in the same sterile soil, we observed that variation of soil microbial diversity affected the fly emergence and choice oviposition. However, it is largely known that soil microbial communities are strongly driven by soil pedology (Thomson *et al.*, 2015), climate (Bardgett and van der Putten, 2014) as well as agricultural management practices (Henneron *et al.*, 2015). Thus, it raises the question of whether similar results could be obtained using another soil or more complexed microbial communities. For instance, the study of Badri *et al.* (2013) tested soil microbial communities from different agricultural management practices to evaluate whether it could influence the feeding of an aboveground herbivore (*Trichoplusia ni* larvae). The authors showed that insect larval weight was reduced by several soil microbiota, among which all microbiota from organic farming.

During my thesis, we also isolated microbial communities from soils of different agricultural management practices (i.e. conventional, organic and cover cropping systems; Henneron *et al.*, 2015) that we used as inocula in the same sterile soil from La Gruche. We tested whether these microbial communities could differently modulate *D. radicum* phenotype (Appendices, paper 5). We observed significantly less damages on the roots grown on soil where microbial communities from the organic system were added compared to the other two, while survival and size of *D. radicum* tended to be slightly lower, though not significant, when developing on plants grown in a soil with microbial communities from organic system compared to microbial communities from conventional system.

These results raise the question of whether the obtained fly phenotype was consistent with our first findings (Lachaise *et al.*, 2017). Results of these two studies can be potentially compared between soil microbial communities from the conventional system (Appendices, paper 5) and soil communities associated to the high soil microbial diversity (Lachaise *et al.*, 2017). It seemed that flies associated to the high soil microbial diversity had an emergence rate two-fold lower and a slightly smaller size (i.e. a 3-4% decrease) compared to flies associated to the conventional system. It should be noted that both studies used the same plant genotype (*B. napus* L. subsp. *oleifera* cv. Tenor) and the same fly population collected in a field in Le Rheu in 2014. Even though the same fly population was used in several but similar experiments, we observed a certain variability for several life history traits (table 1).

Table 1 Variation of life history traits among *D. radicum* populations used during this thesis.

<i>Delia radicum</i> population [†]	Experiment	Mean emergence rate per plant	Mean development time (days)	Mean survival time (days)
“LR14” Le Rheu 2014	Chap II - Paper 1 (Lachaise <i>et al.</i> , 2017)	42.2%	35-40	NA
	Test prior to paper 5	40.1%	42	7
	Appendices - Paper 5	51.5%	39	7
	Functional experiment associated to paper 5	65.7%	39	7
“LR15” Le Rheu 2015	Chap III - Paper 2 (Ourry <i>et al.</i> , 2018)	60.6%	35	6
“PM16” Pleumeur 2016	Chap IV - Paper 4	20.8%	46	NA

[†]Location and year of field sampling in Brittany (France).

While it has been largely demonstrated that plant genotype strongly influences plant chemistry (Poelman *et al.*, 2008) but also herbivores (Mooney and Agrawal, 2008), it is usually known that wild insect populations differ from laboratory ones, which originally come from field sampling at one or several locations or years. Even though different laboratory populations are reared in the same controlled conditions for several generations, the fly phenotype can still vary between populations (table 1). Insect phenotype varying between populations may be due to difference in their microbiota (Bili *et al.*, 2016), thus emphasizing the strong influence of genetics on both plants and insects, and consequently on their interactions.

In order to strengthen and generalize the conclusions that we drew from our results, experiments should be conducted on different type of soils while considering variations due to plant genotypes or fly populations. Using different soils would also permit to better understand

the impact of soil microbial communities on plant-insect interactions and potentially to identify key microorganisms involved in this impact, and thus their underlying functions.

3. Plant chemistry, as a connection between plant and soil microbial communities upon root herbivory?

In the second part of this manuscript, we showed that herbivory by *D. radicum* induced chemical modifications in the roots by decreasing most compounds at the peak of plant-insect interactions (i.e. larval stage feeding on the roots) and by increasing others like carbohydrates and sulfur-containing compounds. Root and rhizosphere microbial communities were impacted by such herbivory which increased the abundance of γ -Proteobacteria and Firmicutes and a couple of their affiliated genera (i.e. *Bacillus*, *Paenibacillus*, *Pseudomonas* and *Stenotrophomonas*). We proposed a hypothetical scenario to explain these chemical and microbial changes: a plant suffering from herbivory would emit defensive (i.e. GSLs) and/or nutritive (i.e. carbohydrates, polyols and organic acids, CPOs) compounds that would influence the recruitment of soil microorganisms by the roots, which in turn would provide new nutrients (i.e. sulfur mineralization) to the plants in order to keep producing sulfured-defensive compounds (GSLs). However, we lacked substantial proofs that these chemical compounds were responsible for the observed microbial changes and further experiments would be necessary to know if root exudates, root volatiles or microbial volatiles are involved.

Root exudates are often considered as a key element in plant-soil microorganism interactions (Haichar *et al.*, 2014; van Dam and Bouwmeester, 2016; Tsunoda and van Dam, 2017). Root exudates and more globally rhizodeposits can induce a large range of mechanisms that form feedback loops between the roots, their microbiota and soil particles. Such mechanisms include processes associated for example, to the soil physicochemical changes; nutrient mobilization; signal exchanges between and within plant as well as between plant and microorganisms; release of plant defense compounds; modifications of microbial community size, composition and activity (Oburger and Jones, 2018). Insect herbivory can change the composition of root exudates, and therefore modify the root and soil microbial communities that interact with the plant. For instance, the presence of aphids (*Myzus persicae*) was shown to modify pepper (*Capsicum annuum*) root exudates and increased the number of gram-positive rhizobacteria colonizing pepper roots (Kim *et al.*, 2016). However, root exudates can have

different effects on soil microorganisms as showed by Badri *et al.* (2013a), which suggested that the same root exudate compound could act as a positive regulator for some OTUs while also acting as a negative regulator for others.

Another key element important to plant-soil microorganism and even microbial interactions is volatile organic compounds (VOCs). These volatiles are well known for being aboveground cues attracting natural enemies of herbivores such as parasitoids, but they can also be emitted by roots and attract soil microorganisms (Schulz-Bohm *et al.*, 2018). Microorganisms like bacteria and fungi can also produce VOCs (Schulz-Bohm *et al.*, 2017), that have various effects and mediate microbial positive, neutral or negative interactions (chapter I figure 1). In our study, we observed that *D. radicum* herbivory induced an increase of some carbohydrates and sulfured-compounds, which was associated with a relative abundance increase of γ -Proteobacteria and Firmicutes such as *Bacillus*, *Paenibacillus*, *Pseudomonas* or *Stenotrophomonas* in the roots and rhizosphere of *B. napus*. We suggested that these bacteria may have been attracted by the chemical compounds emitted by the plant. In the study of Kim *et al.* (2016), it was demonstrated that growth of rhizobacteria *Paenibacillus polymyxa* was promoted by aphid-influenced root exudates. Similarly, Schulz-Bohm *et al.* (2018) showed that *Carex arenaria* roots produced specific root VOC blend upon fungal infection, which resulted into attracting potentially beneficial bacteria like *Paenibacillus*. While it appeared in the study of Kong *et al.* (2016) that whitefly herbivory tended to attract *Pseudomonas* in the rhizosphere, no chemical analysis was performed to explain this result. However, the increase of bacterial abundance in our study may also result from microbial interactions that change following herbivory. For instance, VOCs produced by *Bacillus amyloliquefaciens* applied on soil modified soil microbial communities, where relative abundance of Proteobacteria, Firmicutes, Bacteroidetes and Ascomycota increased while of Acidobacteria and Actinobacteria decreased (Yuan *et al.*, 2017). These authors also observed that microbial VOCs could even alter the expression of genes associated to antibiotic production. Indeed, microbial VOCs can have beneficial or detrimental effects on other microorganisms, either promoting their growth, their antibiotic or antifungal properties for examples, or directly modifying transcriptional expression of genes (Schulz-Bohm *et al.*, 2017).

In order to understand how *D. radicum* herbivory influence its host plant and root and rhizosphere microbial communities, it is necessary to disentangle plant from microbial chemical compounds by dissociating plant and microbial compartments. To achieve this objective, chemical compounds emitted by control and infested plants, for example root

exudates or root and microbial VOCs, could be collected in sterile conditions and applied on other plant roots or unplanted soil, to assess how their associated microbiota react toward plant chemistry influenced or not by insect herbivory. The use of EcoFAB (Ecosystems FABrication), recently developed, could provide the experimental conditions to study plant–microorganism interactions and the mechanisms involved within specific environmental conditions (Zengler *et al.*, 2019), thus with a sterile plant growth platform that includes the EcoFAB device and sterile plant-sized transparent container (figure 2).

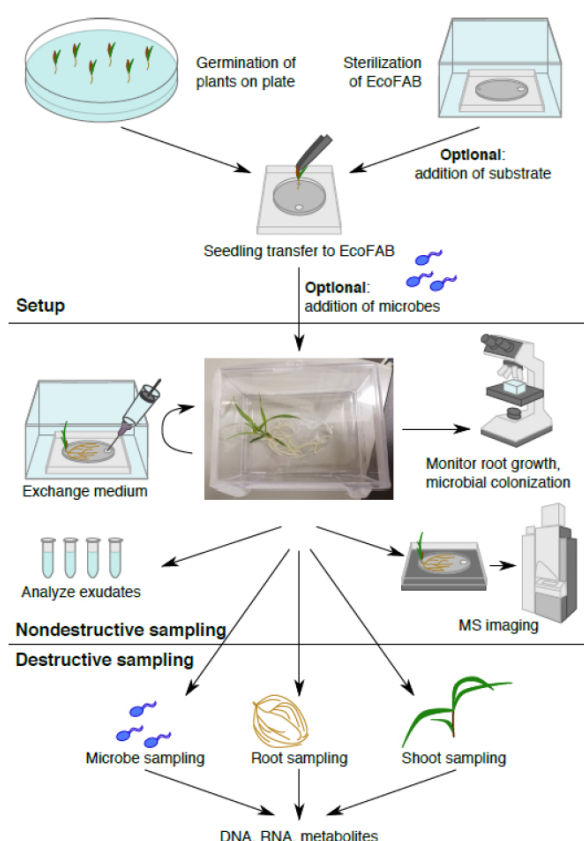


Figure 2 The EcoFAB workflow (from Gao *et al.*, 2018). Plants are germinated on a plate, and transferred to the sterilized EcoFAB where microbes can be added. Nondestructive sampling: root exudates can be sampled and imaged, and root morphology can be visualized. Destructive sampling allows analysis of microbe, root, and shoot parameters in detail.

4. Bacterial legacy in insects

In the third and last part of this manuscript, we showed i) that *D. radicum* bacterial communities changed but were not destroyed when exposed to antibiotic, which effects lasted over several generations, and that ii) several bacterial species seemed to be shared between different development stages of *D. radicum* and between *D. radicum* and *B. napus*.

4.1. Repercussion of insect microbiota on life history traits

Insects harbor symbionts, which can be part of more or less complex microbial communities. Usually, symbionts are studied in order to assess their effects on the life history traits (LHTs) of the insect host. So far, symbionts effects have been largely studied especially in aphids: some increase their host fecundity or longevity, while other promote their host resistance toward abiotic or biotic stresses, like heat, parasitoids or pathogens (Guo *et al.*, 2017). While antibiotics have been generally used for that purpose: eliminating insect symbionts to assess the obtained phenotype, their effects on insect microbial communities were rarely evaluated.

Our study showed that direct application of tetracycline decreased bacterial diversity in flies (but not to the point of turning them into sterilized insects) and induced a shift in the bacterial composition, in terms of both abundance and frequency. Such effects were still visible two generations later on untreated offspring, which parents and/or grandparents were antibiotic-treated. Flies with antibiotic history tended to share bacterial genera that may be potentially tetracycline-resistant and transmissible to the next generation.

Consequently, our results raise the question of how two generations of antibiotic treatment affect the phenotype of the third and untreated generations of *D. radicum*. The same experimental design (figure 3A) as the one described in paper 3 was used to assess LHTs of untreated adult flies which differed by their antibiotic history (Lopez, 2018). As several studies showed that males and females have different bacterial communities (Simhadri *et al.*, 2017; Yong *et al.*, 2017) and that we observed slight differences in our study, LHTs were represented for each sex (figure 3B-D). Tetracycline application tended to increase the time of development, with the three antibiotic history treatments being similar and males developing faster than females (figure 3B). Antibiotics seemed to decrease female survival compared to the control, thus reducing differences between male and female survival (figure 3C). Lastly, female size was homogenous among treatments compared to males, which tended to be smaller when their parents were treated but bigger when their grandparents or both grandparents and parents were treated, than females and control males (figure 4D). Interestingly, we noted that the fly LHTs varied between females and males but that there was only slight differences in terms of microbial communities.

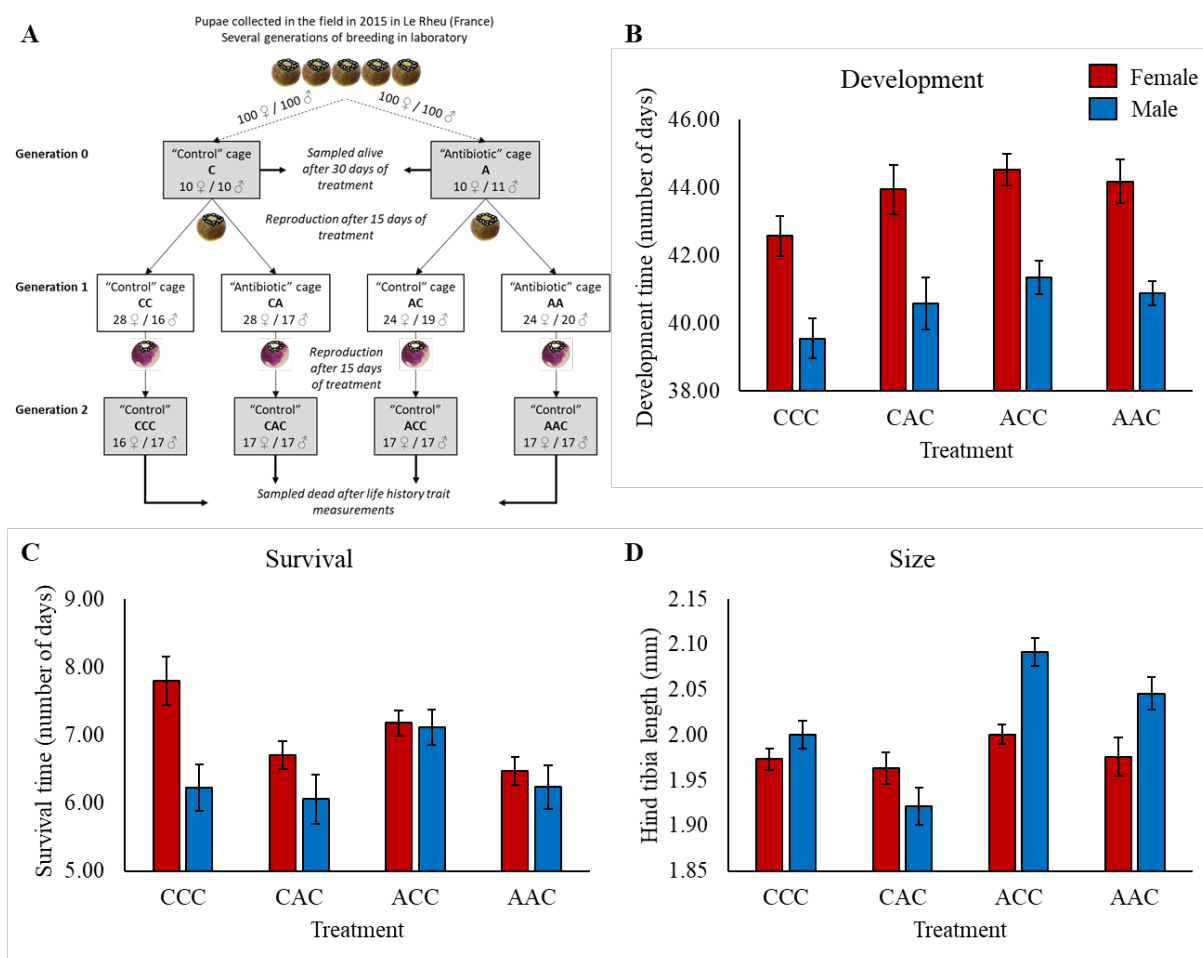


Figure 3 Impact of antibiotic history on the life history traits of the untreated-fly generation (i.e. generation 2). (A) Overview of the experimental design presented in paper 3. Mean (\pm se) of both female and male (B) development time, (C) survival time and (D) size for each treatment (i.e. antibiotic history or not). Note: no statistical analysis was performed on these data yet.

Development time: number of days from the day where eggs are deposited on the host plant to the day of emergence. Survival time: number of days without food from the day of emergence to the day of death.

We observed that both bacterial communities and fly LHTs of the untreated generations were still influenced by their antibiotic history and further analysis (i.e. DIABLO) would permit to find potential correlations between bacterial communities and fly LHTs with antibiotic history and to identify the bacteria responsible for the modified fly phenotype. Furthermore, it also questions the transmission pathways of *D. radicum* microorganisms, as to whether they are only inherited from the previous generations or also acquired from the environment, including the host plant.

4.2. Bacterial transmission in insects

Our study showed that bacterial composition varied across the development stages of the cabbage root fly and that several bacterial species such as *Serratia marcescens* and

Acinetobacter guillouiae were assumed to be shared between mothers and eggs. As insect symbionts can be beneficial to their host, focus has been brought to their transmission mode. Symbionts are usually passed on by the mother either internally or externally, where the eggs or embryos are colonized and then communities can evolve during the insect development. On such matter, aboveground insects such as aphids were also frequently studied (see Michalik *et al.* (2014) for example) while studies on belowground insects remain scarce. In our study, we showed that the bacteria *S. marcescens* was present in all female flies and all unsterilized eggs samples, and thus assumed to be transmitted from the former to the latter. As *Serratia* was found to be able to detoxify isothiocyanate (Welte *et al.*, 2016), we hypothesized that females coat their eggs with these bacteria so that larvae could ingest them when hatching and benefit from this detoxification capacity. Analysis of early larval instars would have helped to verify this hypothesis as to whether these bacteria were still present in first and second instar larva but such stages were considered too difficult to recover from *B. napus* roots in our study. However, this hypothesis should be carefully made as it was based only on the three available female samples. Indeed, several samples in our experiment encountered an insufficient sequencing quality and were thus removed from the dataset prior analyzing. Consequently, several adult stages were eliminated as there was no more samples left, which prevented us from comparing larval and the adult stage that followed or two adult generations, to evaluate whether bacterial communities were constant over several generations.

Additionally, we identified *Pseudomonas* as being shared between larval stage upon feeding and the host plant roots. According to Welte *et al.* (2016), several *Pseudomonas* strains were isolated from *D. radicum* larval gut and displayed as *Serratia*, capacity of resistance toward isothiocyanate. We could hypothesize that larvae acquired such bacteria when feeding from the roots, as plant microbiota would be expected to be immune against their host defenses. Such bacteria like *Pseudomonas* may provide *D. radicum* detoxifying capacity in order to withstand the plant toxic defenses and digest the root tissues. However, it was not possible to confirm that the bacteria were really horizontally transmitted as they were absent from starved larvae. Thus, it could be speculated that *D. radicum* larvae temporarily shunt root microbiota to deal with the plant defenses while feeding during the larval stages as these microorganisms may be useless for the next development stages. Unfortunately, the absence of older stages due the sequencing quality issue previously mentioned also prevented us from concluding to a short use of plant microbiota or to a long lasting horizontal transmission. Still, several studies have shown that insects could acquire microorganisms from the soil (Kikuchi *et al.*, 2012), the roots

(Flury *et al.*, 2018) or the leaves (Hannula *et al.*, 2019). A belowground herbivore was for example shown to acquire bacteria from the rhizosphere and benefit from the bacteria by obtaining its capacity to detoxify insecticides (Kikuchi *et al.*, 2012).

Moreover, horizontal microbial transmission was also supposed to be assessed by comparing the two reproducer generations, which were reared on different host plants. However, samples associated to the adult stages reared on *B. napus* were removed from the dataset due to their poor sequencing quality. Thus, methodology improvement needs to be made to overcome the sequencing quality issues.

However, indications that bacteria are shared between insect stages or between insect and plant do not demonstrate bacterial transmission, as bacteria could be independently present in such insect stage or plant compartment. To confirm the transmission pathway of *D. radicum* bacteria, identified shared bacteria should be labelled and i) followed during the insect development, up to the next generation or ii) inoculated on the host plant and evaluated their acquisition by the insect. Moreover, locating specific bacteria within their insect host may also provide information about their function. For instance, bacteria detected in the gut are expected to hold a function associated to the insect digestion, potentially a detoxifying capacity of plant defenses.

After screening and isolating potentially vertically or horizontally transmitted bacteria, fluorescence microscopy could be a tool used to confirm bacterial transmission pathways in insects. For example, green fluorescent proteins (GFPs) can be combined to microorganisms, and when Kikuchi and Fukatsu (2014) inoculated the soil and budded seeds by watering with GFP-labelled *Burkholderia*, they observed with epifluorescence microscopy the progressive colonization of this symbiont in the gut (main track and midgut crypts) of the bean bug *Riptortus pedestris* (figure 4A). Otherwise, fluorescence in situ hybridization (FISH) can be combined to fluorochrome-labelled oligonucleotides that target bacterial 16S rRNA (i.e. 16S rRNA-targeted FISH) which allows the detection of both cultivable and non-cultivable bacteria. Interestingly, FISH permits to detect simultaneously several different bacteria using different fluorochromes, as showed by Koga *et al.* (2009) which used different probes to detect host aphid nuclei and both of its primary and secondary endosymbionts (i.e. *Buchnera* and *Serratia* respectively, figure 4B) at different developmental stages of the insect.

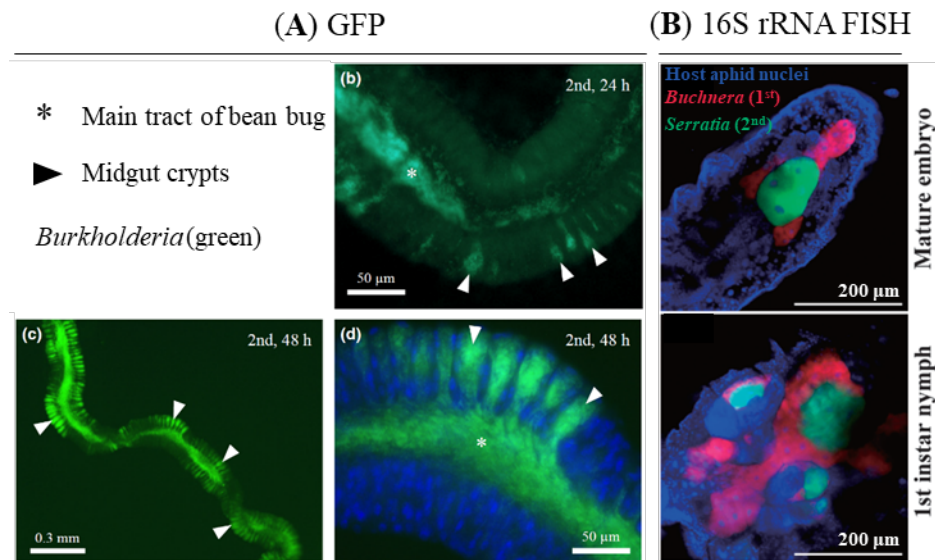


Figure 4 Overview of different fluorescent methods to localize microorganisms. (A) Green Fluorescent Proteins (GFP) showed the progressive colonization of the bean bug gut by *Burkholderia* (modified from Kikuchi and Fukatsu, 2014); (B) 16S rRNA-targeted Fluorescence In Situ Hybridization (FISH) detected host aphid nuclei and its primary and secondary endosymbionts without autofluorescence of the insect tissues at two development stages (modified from Koga *et al.*, 2009).

5. Getting closer to functional approaches

In chapter II, we showed that soil microbial diversity could influence the fly phenotype, but could not be linked to the plant chemistry. To understand how soil microbial communities could modify a belowground herbivore, a possibility would be to evaluate the functions associated to these soil microbial communities using an integrative approach: metatranscriptomic. While this approach allows the profiling of whole gene expression of complexed microbial communities, microbial mRNA is difficult to recover as it corresponds to a very low fraction of the total sequenced RNA, dominated by the host plant RNA. Further optimization of laboratory protocol and bioinformatical tools are required beforehand though.

In chapter IV, we suggested that most remaining bacteria not suppressed by antibiotic application and potentially transmitted to the next fly generation were tetracycline-resistant (i.e. *Serratia* and *Pseudomonas*, the Enterobacteriaceae family) on the one hand. We also proposed on the other hand, that the vertically and horizontally transmitted bacteria (*Acinetobacter*, *Serratia* and *Pseudomonas*) we identified had a detoxification capacity toward GSL-hydrolyzed isothiocyanates so that *D. radicum* could successfully develop. However, these two studies were not designed to assess the functions of microorganisms as only a metabarcoding approach was used.

A bioinformatical tool was developed so that relationships between taxonomic and functional data could be established. PICRUST (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) is a tool that use an algorithm to predict the functional composition in a microbial community from biomarkers genes such as 16S rRNA gene and reference genome database (Langille *et al.*, 2013). Such prediction is based on the relationship between the phylogenetic relatedness of organisms and their complement of functional genes. The tool accuracy was developed by testing PICRUST on microbial data from different hosts and environments (e.g. humans, soils, mammalian guts). Interestingly, PICRUST has the advantage to identify cases where distantly related organisms share the same function (Douglas *et al.*, 2018). These authors provide an in-depth description of how PICRUST should be used. PICRUST was developed using 16S, which is actually limited in term of taxonomy precision as it gives information down to the genus level. However, PICRUST outcome would be more accurate if applied on data sequenced with *gyrB* or *rpoB* primers, which inform about taxonomy down to the species level. While we did not use such tool in our work, it is certain that it would have enriched our hypotheses about the roles and functions of microorganisms in plant-insect interactions. Yet, it is important to retain that PICRUST remains just a predictive tool and does not identify functions.

Microbial communities can be differently assessed depending on the research question, and thus the method used to answer it. The different papers presented in this manuscript showed that we manipulated microbial communities of either soil or insects in order to study more simplified communities and so we could further hypothesize about their role in plant-insect-microorganism interactions.

“Destructive” methods were used in this manuscript in order to simplify the microbial communities of interest. For instance, we used the dilution to extinction method to obtain a decreasing gradient of soil microbial diversity in chapter II, also applied in the study of (Hol *et al.*, 2010), and evaluate the impact of *D. radicum* LHTs. We also used an antibiotic on *D. radicum* in chapter IV, which was expected to eliminate most of the insect bacterial communities, to further assess the effect of altered bacterial communities on their insect host LHTs.

“Creating” methods can also be used and is usually applied to test hypotheses about identified microorganisms or their associated functions. For example, synthetic communities (SynComs) are “created artificially by co-culturing of select (two or many) species under a (at least initially) well-defined media” in order to assess the ecological, structural and functional

features of microbial communities in controlled conditions (Großkopf and Soyer, 2014). To create SynComs, microbial screening and isolation should be first performed on one or several compartments of one or several plant genotypes or species. Then, selection of microorganisms should be done according to microbial pairwise combinations performed to assess the interaction nature (i.e. antagonist, mutualist...) and according to the research question. Lastly, the creation of a simpler SynCom is made from the previously selected and identified microorganisms (Großkopf and Soyer, 2014). Different experimental systems exist to test the effect of SynComs on plants: SynCom-inoculated agar medium; the calcined clay system (figure 5A, Bai *et al.*, 2015) and the FlowPot system (figure 5B, Kremer *et al.*, 2018), all under sterile conditions. The equivalent method for insect would either be the creation of free and symbiont-infected insect lines through reproduction (Lopez *et al.*, 2018) or combination of destructive and creating methods, with antibiotics to eliminate insect symbionts and injection of new one(s) (Leclair *et al.*, 2017).

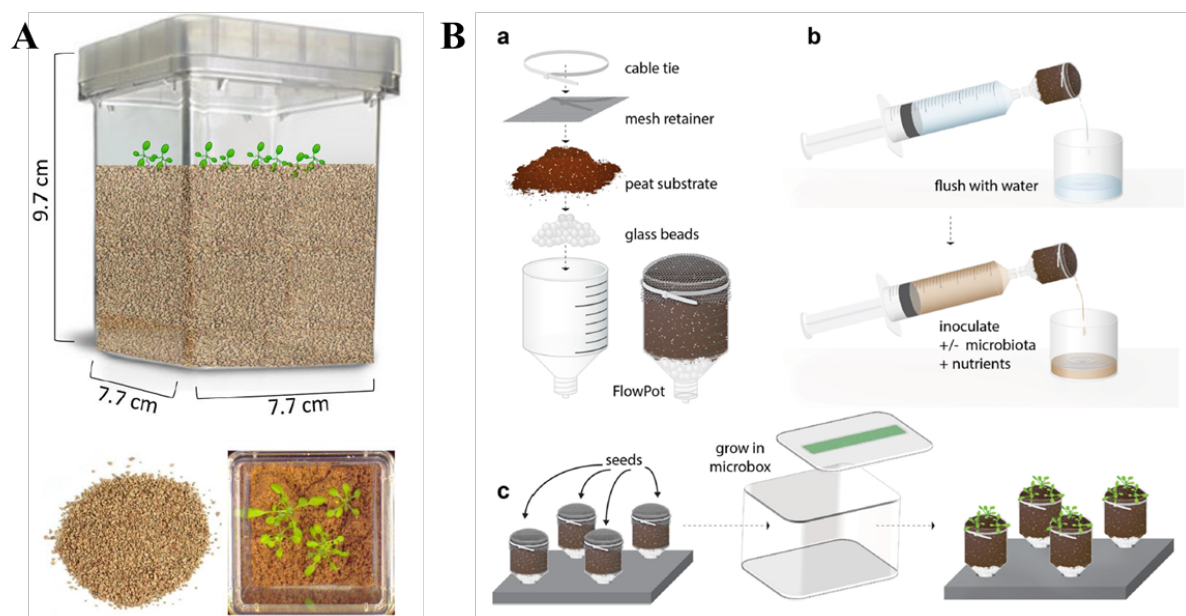


Figure 5 Experimental systems used to study SynComs. (A) calcined-clay (figure from EMBO “Plant microbiota” practical course). (B) FlowPot can be completed in three steps: (a) implementation of the FlowPot, (b) sterilization and inoculation with the SynComs, (c) sowing and box sealing (figure from Kremer *et al.*, 2018).

Therefore, manipulating microbial communities permits to unravel the role of simplified communities within plant-insect interactions but the next step would be to further confirm our hypotheses about the potential role of identified microorganisms associated to *B. napus* or *D. radicum*.

Perspectives

The present work aimed at assessing the impact of soil microbial communities on plant-insect interactions, determining how insect herbivory influences back these communities through the plant chemistry and evaluating the bacterial communities of a belowground herbivore. Based on our results, and as we just saw, several research perspectives can be proposed in order to identify the role of plant defenses and of microorganisms toward a belowground herbivore.

- Determining how plant defenses are modulated by soil microorganisms and identifying the plant compound(s) responsible for changing insect life history traits;
- Strengthening our results by experimenting on several soils, plant genotypes and insect populations;
- Characterizing the plant mechanisms that modify root and soil microbial communities upon insect herbivory;
- Pinpointing insect bacterial transmission by labelling the shared bacterial species we observed;
- Assessing the functions of microorganisms associated to soil, plant and insect in order to better understand plant-insect interactions.

Although the results presented in this manuscript are still far from leading to field applications, it is essential to know the possibilities of these field applications that lay ahead of fundamental researches, in order to take most considerations into account to facilitate the transfer of lab results into field conditions.

It is challenging to apply microbial results obtained from the laboratory in field conditions as multiple cases emerged where microbial inocula have no effect in the field despite having shown positive results in the laboratory. To optimize the application of laboratory knowledge into field conditions, several aspects have to be considered (Sessitsch *et al.*, 2019, figure 6). In order to obtain a successful establishment of the microbial inoculum and its desired effects in a new and natural environment, appropriate number of cells and suitable formulation (e.g. encapsulation to protect the microorganisms) should be applied while considering the compatibility of the inoculum with the target plant and its adaptation to the environment biotic and abiotic conditions. The latter implies that the microbial inoculum must bear and go through

competition with the resident microorganisms, which makes evaluation of microbial interactions essential.

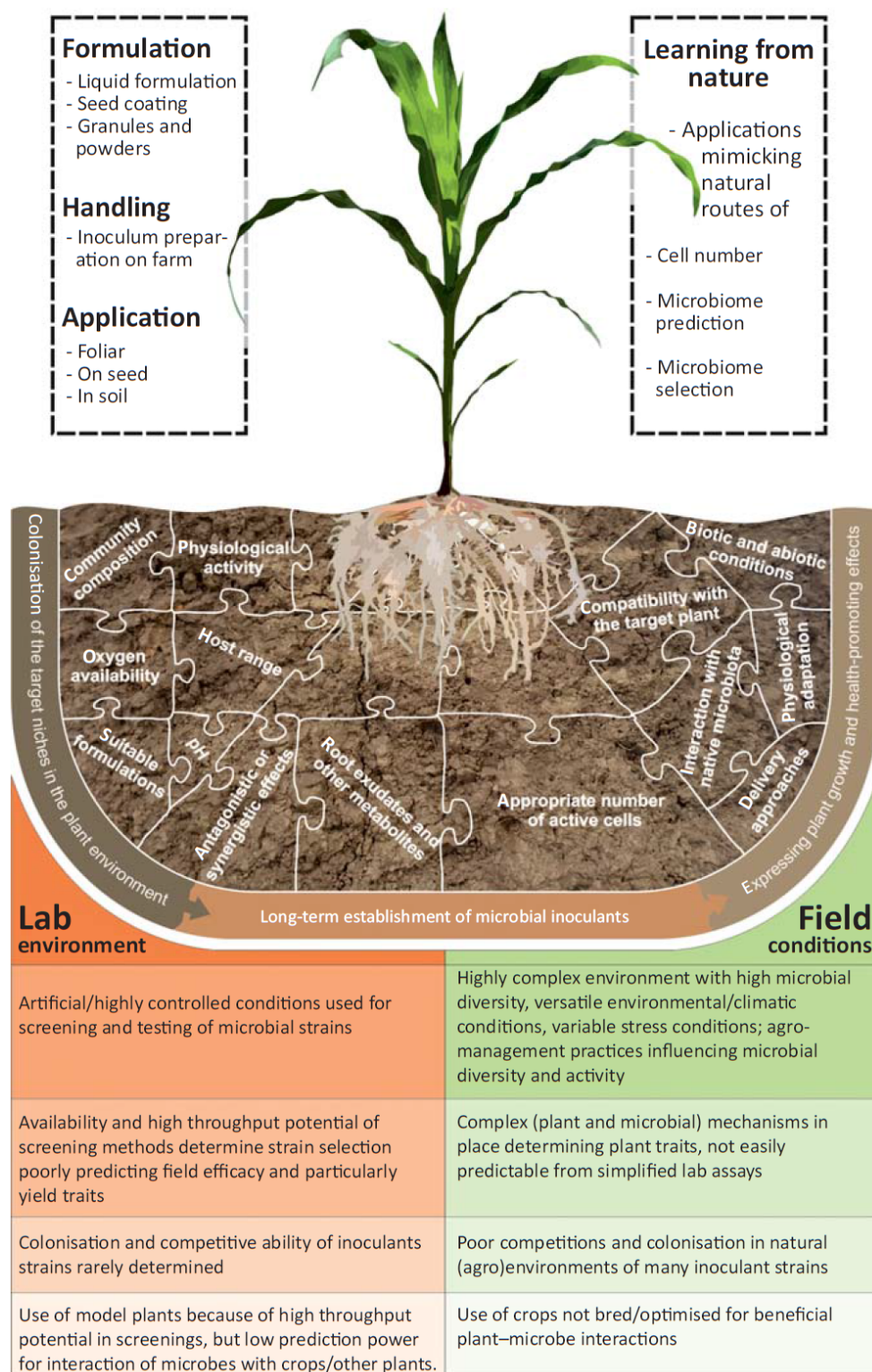


Figure 6 Challenges of Microbial Inoculation (from Sessitsch *et al.*, 2019)

Once microorganisms have been identified as toxic to insects or as boosting plant defenses, different strategies can be carried out in order to use microorganisms for biological control. For instance, one of the most well-known biological insecticides used in France is composed of one bacterial species: *Bacillus thuringiensis* (also called Bt). This insecticide is toxic to insects when ingested and is usually sprayed on plant leaves during insect infestation (Monnerat *et al.*, 2009; Sanchis, 2016). Entomopathogenic fungi are also promising in insect biological control, like the Naturalis product made out of the fungus *Beauveria bassiana*, that can also be sprayed on leaves (Klieber and Reineke, 2016).

While individual species or their product can be interesting for plant protection, our results point toward effects at the community level. New techniques have been developed that allow whole microbial communities to be inoculated at once with seeds acting as a protective shield for these microorganisms. For example, “EndoSeed” is a method where a liquid solution containing microorganisms is sprayed over the plant during the flowering so that microorganisms could later colonize the emerging seeds (Mitter *et al.*, 2017, figure 7.1). “SEEDJECTION™” (www.seedjection.at) is an automated and continuous mechanical method, which performs a cut on the seed without harming the embryo in order to inject a microbial solution before closing back the seed surface (figure 7.2). Seed coating is defined as “a reliable technique to apply exogenous materials (such as biopolymers, colorants, biocontrol agents and microbes) in close proximity with germinating seeds, which ultimately improves seed quality (viability and vigor) and yield through enhancing the seed placement and performance” (Ma, 2019).

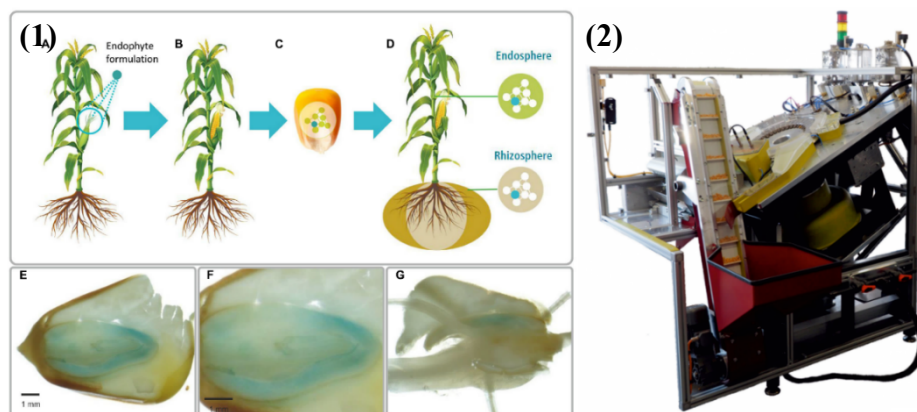


Figure 7 Illustration of two methods to introduce plant beneficial bacteria into plant seed: (1) “EndoSeed” (Mitter *et al.*, 2017) and (2) “SEEDJECTION™” (from www.seedjection.at). (A–D) Plant flowers are sprayed with a bacterial suspension, which bacteria colonize flowers and the developing seeds. Mature seeds are collected and endophytes stay viable during seed storage. Endophytes proliferate during germination and colonize the offspring plant generation. Light microscopy images of a mature maize colonized by *Paraburkholderia phytofirmans* strain PsJN::gusA (E–G). The blue is due to GUS-stained bacterial cells. Strain PsJN is present inside the embryo (E,F) and in radicals (G).

While several studies have demonstrated the capacity of these methods to promote plant growth, emerging studies focused on biological control of pathogens (Ma, 2019) and very few on insects, but no doubt that such technologies hold promising results for the future.

Conclusion

The originality of the present work comes from the fact that we focused on the interaction between a crop plant (*Brassica napus*) and a root herbivorous insect (*Delia radicum*), while considering their associated microbial communities, thus making a tripartite interaction or an interaction between two holobionts that we studied over three chapters.

We showed that soil microorganisms can indeed be used as a leverage to influence the development and behavior of a root herbivorous insect, which feeding was demonstrated to be a strong driver of the plant chemistry and microbial communities. Though the use of antibiotic has long lasting effects on the insect microbial communities and phenotype, remaining bacteria are suspected to be successfully transmitted over the generations, potentially contributing to insect survival. While vertical and horizontal transfers of microorganisms contribute to shaping *D. radicum* microbial communities, we speculate about transmitted bacteria being involved in the detoxification of plant defenses.

Having a better understanding of plant-insect interactions and how strongly microorganisms can influence their hosts or other interacting organisms is a crucial step that could promote microbial applications and their use in innovative and environmental friendly biocontrol methods to manage insect pests.

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APPENDICES



APPENDICES

Paper 5

Root herbivore development improved by microbial communities from organic system and soil-borne pathogen

In construction

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Author contributions

SD, CM, LL and MO conceived and designed research. AYGE, BM, LL, MO and SD prepared the soil used in the experiment, cultivated the plants and performed sampling. AYGE, BM, CL, CM, CP, LL, MO and SD measured the fly phenotype. MO did the statistical analyses and interpreted data. MO wrote the manuscript that was commented and approved by all authors.

Key message

- *Brassica napus* root symptoms resulting from the rhizophagous insect *Delia radicum* at both larval and emergence stages, were less frequent on soil microbial diversity that originated from either organic or conventional agricultural practices compared to the cover cropping one.
- The presence of *Plasmodiophora brassicae*, a soilborne pathogen and agent of the clubroot disease, accentuated the plant diseased phenotype, worse in soil microbial diversity from organic and cover cropping agricultural practices.
- The pathogen improved the development of *D. radicum* by decreasing its development time and increasing its fitness, with a higher survival time and hind tibia length but slightly decreased emergence level, especially in soil microbial diversity that originated from an organic agricultural practice.

Keywords: Soil microbial diversity, agricultural practices, *Brassica napus*, *Delia radicum*, *Plasmodiophora brassicae*, plant phenotype, life history traits.

1. Preamble

The experiment presented in this paper is a phenotype-centered and a side experiment of the “IMPRESS” project, which objective is to evaluate the “Impact of the plant Microbiota interactions on Plant RESponse to co-infection by two rhizosphere pestS” using a metatranscriptomic approach.

In field conditions, Rathore *et al.* (2017) showed that agricultural practices shaped differently oilseed rape microbial communities depending on the tillage method used. Moreover, co-infections are most likely to occur and coexistence of different bioagressors on the same host plant is possible but quite variable depending on the location as showed by Vojtěch *et al.* (2017) with *Delia radicum* and *Plasmodiophora brassicae* interaction.

The present side-experiment is phenotype-centered as the aim was to assess the impact of soil microbial diversity modulated by different agricultural practices on the development of the rhizophagous insect (*D. radicum*), and determine whether the presence of a soil-borne pathogen (*P. brassicae*) affects the insect development. To do so, we evaluated i) *Brassica napus* phenotype by measuring leaf and root dried biomasses as well as *D. radicum* symptoms on the plant roots and the overall plant health, and ii) the fly phenotype by monitoring the emergence dynamics, calculating emergence rate, assessing development and survival times, and measuring adult size.

2. Materials and Methods

2.1. Soil preparation, inoculation and recolonization

The soils used in our experiment were collected on September 7th 2018 from the layer – 10 to –30 cm at the experimental station of “La Cage”⁷, Versailles (France), which wheat crop is monitored yearly since 1997 (Henneron *et al.*, 2015). Three soils were of interest as they differed from the agricultural management or practice used: the organic (“ORGA”) system, the conventional (“CONV”) system and the cover cropping (“COVER”) system (table 1). Each soil type was sampled from two 0.5 ha fields and pooled together, before manual, 5 mm and 2 mm sieving.

⁷ <http://www.versailles-grignon.inra.fr/en/All-the-news/La-Cage-trial>

Table 1 History of the soils used in our experiment that originate from the organic (“ORGA”), conventional (“CONV”) and cover cropping (“COVER”) agricultural practices.

	ORGA	CONV	COVER
Characteristics	The organic system satisfies the specifications (cahier des charges) of biological agriculture. In the absence of breeding, fabaceous plants insures the nitrogenous nutrition of the crops.	The conventional system, which is based on the achievement of high yield, uses very productive varieties and high quantities of nitrogen and pesticides.	The cover cropping system is based on the suppression of tillage/soil management and the maintenance of a cover plant including during the principal crop cycle.
Previous crop	Wheat	Wheat	Wheat
Current crop	Oilseed rape	Oilseed	—
Tillage	No	Yes	No
Herbicide application 1 to 2 weeks before sampling	Yes	Yes	—
Molluscicide application	Yes	No	—

Samples of these original soils were sent to the LAS (Laboratoire d’Analyses des Sols d’Arras, INRA, France) to assess their main physicochemical characteristics while the number of bacterial and fungal colony forming units (CFUs) were evaluated by growing bacteria and fungi on TSA and malt media. Until experiment, the soils were then stored in the dark at ambient temperature.

Following the protocol of Lachaise *et al.* (2017) and Ourry *et al.* (2018) and according to figure 1, 100 g of each soil diluted in 500 mL permutated H₂O was used to inoculate 5 kg of gamma-sterilized soil (1/3 sand, 2/3 soil) from La Gruche (Le Rheu, France, 48°08’44”N, 01°47’97” W). The next day and thus during the first week, each 5 kg opaque container was agitated using a turbula mixer for 5 min and aired under a laminar flow cabinet for 3 min. During the second week, the agitation and airing steps were performed every two days. During the recolonization process, the containers were stored in the dark at 18°C.

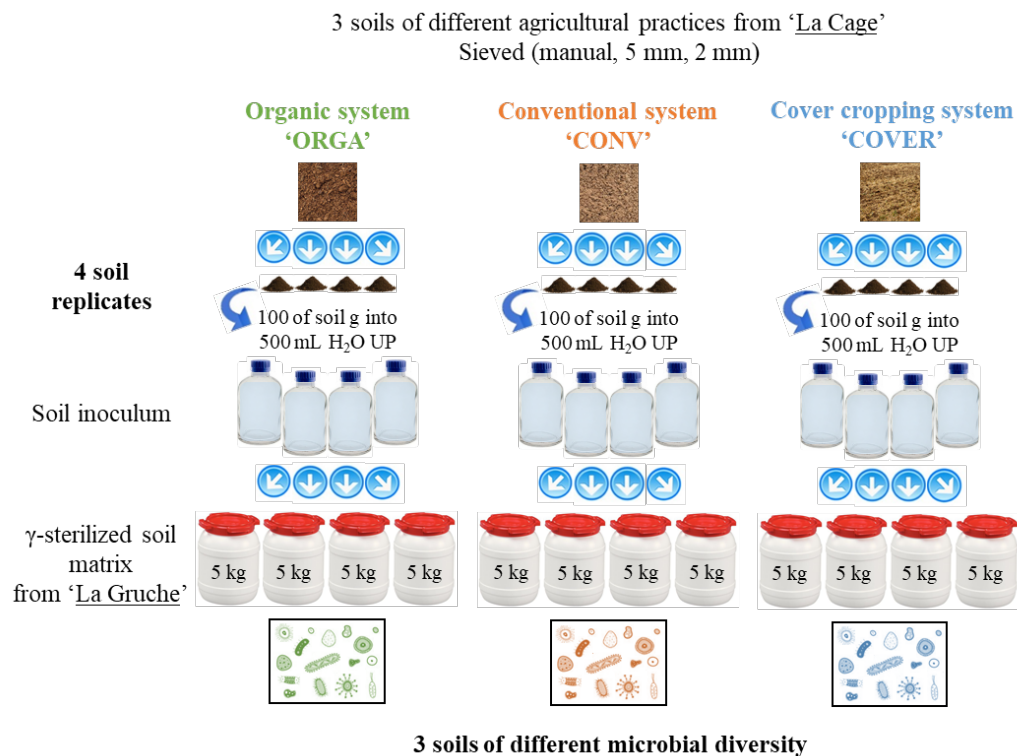


Figure 1 Experimental design for soil inoculation.

The microbial recolonization process was monitored at 1, 2, 7 and 14 days after reinoculation by counting the number of bacterial and fungal CFU and the recolonization was estimated completed when the number of CFUs reached a plateau, allowing the start of the experiment (figure 2). Hence, after two weeks of recolonization, three soil conditions of different microbial diversity were obtained and called: CONV, ORGA and COVER. Sampling was performed on these soils in order to assess the bacterial and fungal diversities, with gyrB and ITS primers respectively, using a metabarcoding approach and MiSeq Illumina sequencing.

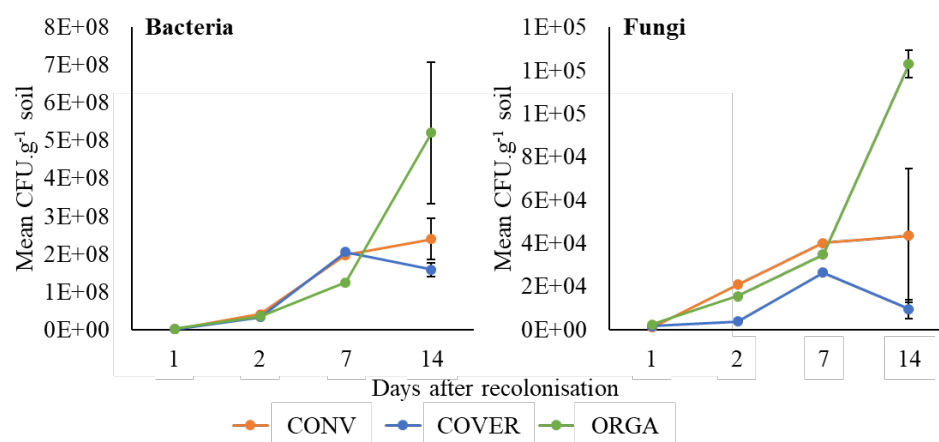


Figure 2 Assessment of microbial recolonization to determine when to start the experiment. N = 3 and 5 Petri dishes per soil treatment at 1, 2 and 7 days for bacteria and fungi respectively but 9 and 15 for 14 days after recolonization.

2.2. Biological models and experimental setup

A layer of pozzolan was placed in a pot with 480 g of soil, which was humidified with a Hoagland solution in sub-irrigation for 24h to reach 100% of water retention capacity (Hoagland and Arnon, 1950; Ourry *et al.*, 2018). Then, oilseed rape seeds (*Brassica napus* L. subsp. *oleifera* cv. Tenor) were sown and cultivated in a climatic room under the following conditions: 16:8 LD, 20:18°C using LED light for three months (figure 3).

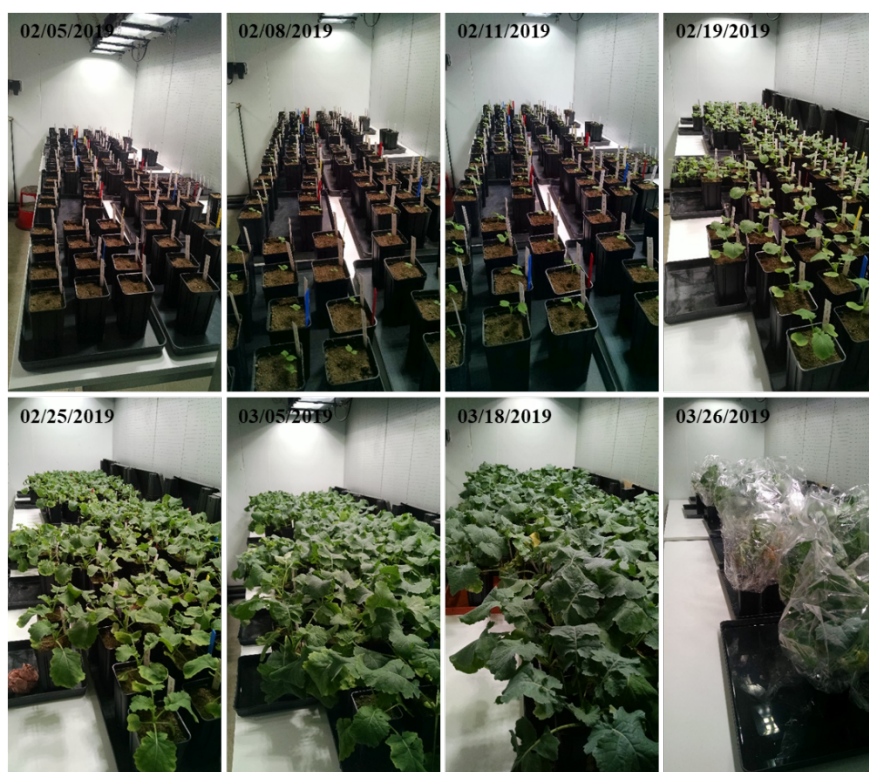


Figure 3 Follow-up of the plant growth in the climatic room. Sowing occurred on the 1st of February, 2019.

2.2.1. Inoculation of *Plasmodiophora brassicae*

Plasmodiophora brassicae (“Pb”) is a soilborne pathogen responsible for the clubroot disease, which largely affects members of the Brassicaceae family. This pathogen life involves three stages: survival in the soil as resting spores, root hair primary infection and secondary cortical infection (Kageyama and Asano, 2009). This process results in the hyperplasia and the hypertrophy of infected roots, leading to the formation of club-shape galls on the root.

Ten days after sowing, the plants were inoculated with 1 mL of Pb inoculum (10^7 spores.mL⁻¹) following the protocol of Lebreton *et al.* (2019). Briefly, the eH inoculum was prepared from three frozen galls, originally sampled on Chinese cabbage (*Brassica rapa*, ECD5) plants, thawed at ambient temperature. Fifteen grams of galls were ground in 100 mL sterile permutated water and filtered on stamen fabric, before being sieved at 500 µm, 250 µm

and 100 µm. After counting the spores in this solution thanks to a Malassez cell, the inoculum was diluted to obtain the right concentration of spores (i.e. 10^7 spores.mL⁻¹). From the inoculation, the plants were watered from the top with a Hoagland solution to maintain a water retention capacity ranging between 70–100%.

2.2.2. Infestation with *Delia radicum*

The cabbage root fly (“CRF”, *Delia radicum*) is a specialist rhizophagous herbivore of brassicaceous plants. Females select host plants based on their recognition of different aboveground physical and chemical characteristics (Gouinguéné and Städler, 2005) and lay their eggs at the base of plant stems. Eggs hatch within a few days and the three larval instars feed by tunneling into the roots for 2–3 weeks, hence damaging massively the roots, before pupating in the nearby soil. Adult emergence occurs about a month after oviposition.

The population used in our experiment was collected in the field in 2014 (Le Rheu, Bretagne, France). In the laboratory, flies were fed on sugar, milk powder and yeast (ratio 1:1:1) and they were reared on rutabaga roots (*Brassica napus* subsp. *rapifera*) in a climatic room (16:8 LD, $21 \pm 2^\circ\text{C}$; $60\% \pm 10\%$ RH) as described in Neveu Bernard-Griffiths (1998). Adult flies were left to oviposit on rutabaga roots for 24h and ‘black-headed eggs’ (i.e. ready to hatch) were collected 3 days later for our experiment.

Sixteen eggs were deposited on the stem base (i.e. crown) of healthy and *P. brassicae*-inoculated plants (20 days after Pb inoculation), hence obtaining two infestation status: “Pb-”, plants infested by *D. radicum* only and “Pb+”, plants inoculated with *P. brassicae* and infested by *D. radicum*. After this infestation, plants were watered through sub-irrigation with a Hoagland solution.

A week before emergence, bags were placed on each plant to collect the emerging flies.

2.2.3. Phenotype measurements

Plant phenotype Leaf and root dried biomasses were assessed for each plant at the end of the fly emergence. Symptoms caused by the CRF on the roots were noted on Pb- plants only at 18 days after infestation (49 days after sowing) when the larvae were currently feeding on the roots, and at the end of fly emergence, hence corresponding to two different sampling times. Symptoms were noted as the proportions of roots (i.e. area from 0 to -5 cm of taproot) damaged by the CRF: I, 0% (no symptom); II, 10-40%; III, 40-90%; IV, 90-100% (dead plants). The overall plant phenotype was evaluated at the end of emergence and noted as follows: 0, leaves

are all dead; 1, most leaves are dead but some are still green, the plant is also smaller; 2, all leaves are green and the plant looks healthy and has a normal size (figure 4).



Figure 4 Scale of the notations applied to assess the overall plant phenotype.

Fly phenotype Starting the first day of emergence, plants were monitored daily at the same time to recover the emerging flies. The following traits were measured: emergence dynamics, as the cumulated number of emerging flies per day (i.e. time factor); emergence rate, as the number of emerging adults divided by the number of eggs deposited on the plant; development time, as the number of days between infestation and emergence; survival time, as the number of days between emergence and death (flies were kept individually in small 125 mL plastic tubes with water only and placed in 96% ethanol when dead for size assessment); hind tibia length, as an index of adult size (measured under a binocular microscope (x100) using “Image Focus” software).

2.3. Statistical analyses

Analyses were performed using the R software (R Core Team, 2018) and a 5% threshold for statistical significance.

On each model (table 2), an analysis of variance was performed (“Anova” function, “car” package) to assess the effect of the different factors used in the model (Fox and Weisberg, 2011). When a factor was significant, pairwise comparison were realized with the “emmeans” and “CLD” functions from the “emmeans” package (Lenth, 2019). Plotting were either performed using the basic functions in R or using the “ggplot” function from the “ggplot2”

package (Wickham, 2016) and the “gridExtra” package for annotations (Auguie, 2017). P values were corrected using the “false discovery rate”.

Table 2 Statistical analyses and models used for plant and fly phenotypes.

y	Statistical analysis	Model	Function Package
Leaf dried biomass	Linear Mixed-Effects Model	y ~ Initial soil microbial diversity x Infestation status + (1 Soil replicate)	lmer, “lme4” ¹
Root dried biomass	Linear Mixed-Effects Model	sqrt(y) ~ Initial soil microbial diversity x Infestation status + (1 Soil replicate)	lmer, “lme4”
<i>D. radicum</i> symptoms	Cumulative Link Model	y ~ Initial soil microbial diversity + Sampling time + Soil replicate	clm, “ordinal” ²
Plant phenotype	Unfeasible as the results are not discriminant enough		
Emergence dynamics [†]	Generalized Linear Model (binomial family)	y = cbind (cumulated nb of emergents, cumulated number of non emergents) y ~ (Initial soil microbial diversity + Infestation status + Time) ² + Soil replicate)	glm gather, “tidyr” ³
Emergence rate	Generalized Linear Mixed-Effects Models (binomial family)	y = cbind (nb of emergents, nb of non emergents) y ~ Initial soil microbial diversity x Infestation status + (1 Soil replicate)	glmer, “lme4”
Development time	Generalized Linear Mixed-Effects Models (gamma family)	y ~ Initial soil microbial diversity x Infestation status + (1 Soil replicate) + (1 Plant_ID)	glmer, “lme4”
Survival time	Regression for a Parametric Survival Model	y ~ Initial soil microbial diversity x Infestation status + Soil replicate	Surv, survreg, “survival” ⁴
Hind time length	Linear Model	y ~ Initial soil microbial diversity x Infestation status + (1 Soil replicate) + (1 Plant_ID)	lm

[†] This analysis was performed twice, considering time as a quantitative to assess the global emergence dynamics, then as a qualitative variable in order to compare modalities at a given time.

¹ (Bates *et al.*, 2015); ² (Christensen, 2019); ³ (Wickham and Henry, 2019); ⁴(Therneau, 2015)

3. Results

3.1. Soil

Original soils The main physicochemical characteristics of the three soils sampled in “La Cage” are displayed in table 3. The soils from the ORGA and CONV systems had similar properties while the soil from COVER was slightly different as the amount of coarse silt seemed to be lower and the amount of fine and coarse sands higher compared to the other two soils. Moreover, COVER soil was associated with higher levels of organic matter, potassium, sulfur and nitrogen-containing nitrates.

Table 3 Physicochemical characteristics (mean \pm se) of the soils used in our experiment that originate from the organic (“ORGA”), conventional (“CONV”) and cover cropping (“COVER”) agricultural practices. Bold writing corresponds to either higher or lower level of compounds in the UVC soil. N = 3 replicates per soil.

Compounds - unit	ORGA	CONV	COVER
Clay (<2 μ m) - g/kg	175.67 \pm 0.67	173.67 \pm 1.67	159 \pm 1.53
Fine silt (2/20 μ m) - g/kg	190.33 \pm 0.33	194.67 \pm 2.03	177 \pm 1
Coarse silt (20/50 μ m) - g/kg	427 \pm 0.58	412.67 \pm 1.76	313.67 \pm 1.33
Fine sand (50/200 μ m) - g/kg	185 \pm 0	196 \pm 1.53	291 \pm 1.15
Coarse sand (200/2000 μ m) - g/kg	22 \pm 0	23 \pm 0.58	59.33 \pm 0.88
Organic C - g/kg	10.2 \pm 0.06	11.1 \pm 0.15	13.4 \pm 0.06
Total N - g/kg	0.99 \pm 0	1 \pm 0	1.32 \pm 0.01
Total CaCO ₃ - g/kg	< 1	< 1	< 1
MO - g/kg	17.57 \pm 0.09	19.2 \pm 0.25	23.2 \pm 0.1
C/N	10.33 \pm 0.07	11.13 \pm 0.12	10.17 \pm 0.03
pH	6.75 \pm 0.01	7.19 \pm 0.02	6.83 \pm 0.01
P ²⁰⁵ - g/kg	0.1 \pm 0	0.14 \pm 0	0.13 \pm 0
CEC [†] - cmol +/Kg	9.11 \pm 0.09	9.53 \pm 0.02	9.89 \pm 0.06
Ca - cmol +/Kg	8.54 \pm 0.01	8.89 \pm 0.1	9.52 \pm 0.03
Mg - cmol +/Kg	1.07 \pm 0.01	1.44 \pm 0.01	1.21 \pm 0.01
Na - cmol +/Kg	0.03 \pm 0	0.03 \pm 0	0.03 \pm 0
K - cmol +/Kg	0.43 \pm 0.01	0.58 \pm 0.01	0.64 \pm 0.01
Fe - cmol +/Kg	< 0.005	< 0.005	< 0.005
Mn - cmol +/Kg	0.04 \pm 0	0.03 \pm 0	0.03 \pm 0
Al - cmol +/Kg	\approx 0.02	\approx 0.02	\approx 0.02
S - mg/kg	168.33 \pm 4.81	176.33 \pm 0.67	216.67 \pm 1.76
N NO ₃ - mg/kg	9.42 \pm 0.09	9.63 \pm 0.73	20.8 \pm 0.1
N NH ₄ - mg/kg	2.83 \pm 0.05	2.96 \pm 0.02	3.2 \pm 0.03

[†] Cation exchange capacity Metson method

Regarding cultivable microorganisms, “ORGA” had less bacterial CFUs than “CONV” and “COVER” while the highest number of fungal CFUs was associated to “CONV” only (figure 5). Moreover and according to visual observations, the aspect of the Petri dishes looked homogenous for both bacteria and fungi in the “CONV” condition in term of spatial distribution and both shape and look of the CFUs. As for the two other conditions (“ORGA” and “COVER”), the spatial distribution looked sparse and there were more filamentous bacteria and fungi.

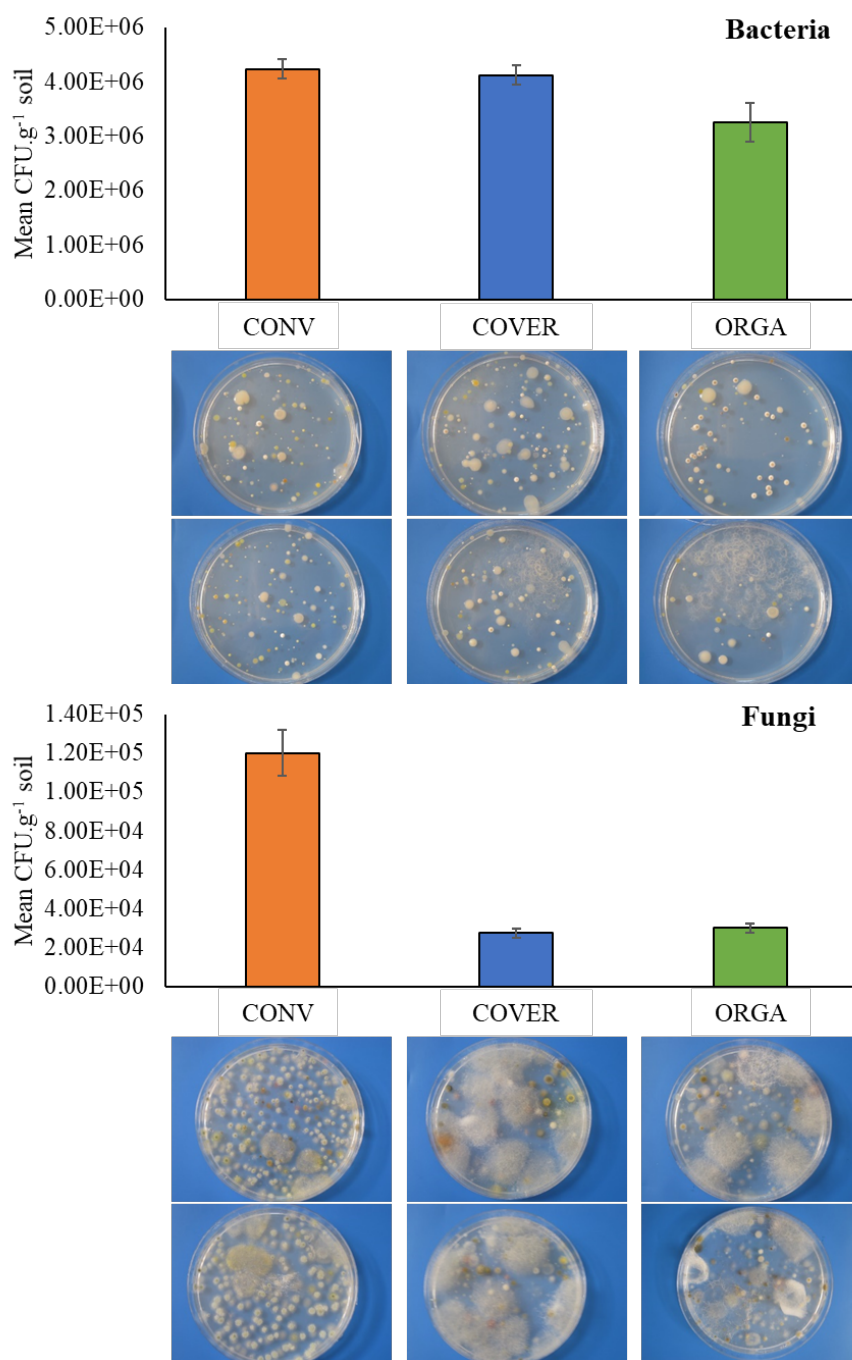


Figure 5 Cultivable bacteria and fungi from the three original soils sampled in “La Cage”. The number of CFUs was counted in Petri dishes, which photos were taken. “ORGA”, “CONV” and “COVER” stand for organic, conventional and cover cropping agricultural practices respectively. N= 9 and 15 Petri dishes per soil treatment respectively for bacteria and fungi.

Manipulated soils At the end of the recolonization, “CONV” and “COVER” reached a plateau with a lower number of bacterial and fungal CFUs than “ORGA”, which however, kept increasing. Just before sowing, these soils were sampled to assess their initial soil microbial diversity. DNA extraction will be performed soon on these samples, using gyrB and ITS primers

respectively, and samples will be sequenced with Illumina MiSeq at the GeT-PlaGe platform (Toulouse, France).

3.2. Plant phenotype

Leaf dried biomass was significantly influenced by infestation status and by the interaction between the latter factor and the initial soil microbial diversity (table 4). The presence of Pb decreased leaf biomass for the three soil conditions (figure 6A). While there was no difference of biomass between soil conditions for Pb-, the decrease of leaf biomass was less important in “CONV” for Pb+, hence being higher than in both “ORGA” and “COVER”.

Root dried biomass was affected by both initial soil microbial diversity and infestation status, as well as their interaction (table 4). As for the leaves, root biomass decreased with Pb but this time the biomass was similar between the three soils for Pb+ (figure 6A). For Pb-, there was a significant decrease of biomass in “COVER” condition compared to “CONV” and “ORGA”, with the latter having the highest root biomass.

Table 4 Outputs of the analyses of variance performed on each plant and fly trait model. P values written in bold indicate a significant effect of the associated factor, also in bold.

Traits	Statistics
Leaf dried biomass	Initial soil microbial diversity: $F=2.05$; $df=2$; $P=0.35$ Infestation status: $F=173.3$; $df=1$; $P<0.001$ Initial soil microbial diversity:Infestation status: $F=6.51$ $df=2$; $P=0.038$
Root dried biomass	Initial soil microbial diversity: $F=11.5$; $df=2$; $P=0.003$ Infestation status: $F=158.3$; $df=1$; $P<0.001$ Initial soil microbial diversity:Infestation status: $F=8.33$; $df=2$; $P=0.015$
<i>D. radicum</i> symptoms	Initial soil microbial diversity: $\text{Chisq}=22.46$; $df=2$; $P<0.001$ Sampling time: $\text{Chisq}=5.10$; $df=1$; $P=0.023$ Soil replicate: $\text{Chisq}=3.49$; $df=3$; $P=0.32$
Overall phenotype	Unfeasible

The presence of CRF symptoms on the plant roots was impacted by initial soil microbial diversity and sampling time (i.e. larval stage vs end of adult emergence, table 4). The plants infested by both Pb and CRF were not taken into account for this trait as there were not enough roots, badly damaged by dual infestation, to assess CRF symptoms. When sampling occurred during the insect larval stage, the symptoms were greater in the “COVER” condition than the other two, “ORGA” having significantly less symptoms (figure 6B). The same pattern was observed when plants were sampled at the end of fly emergence, with however more intermediate symptoms than sampling at larval stage, which had more extreme symptoms.

The notation of the overall plant phenotype could not be analyzed using statistics as the data were not discriminant enough. However, it was quite clear that all plants infested by both Pb and CRF looked sick, and even partially dead or dead (figure 6C). The plants grown on the “COVER” condition seemed to be sicker than on “ORGA” and “CONV”.

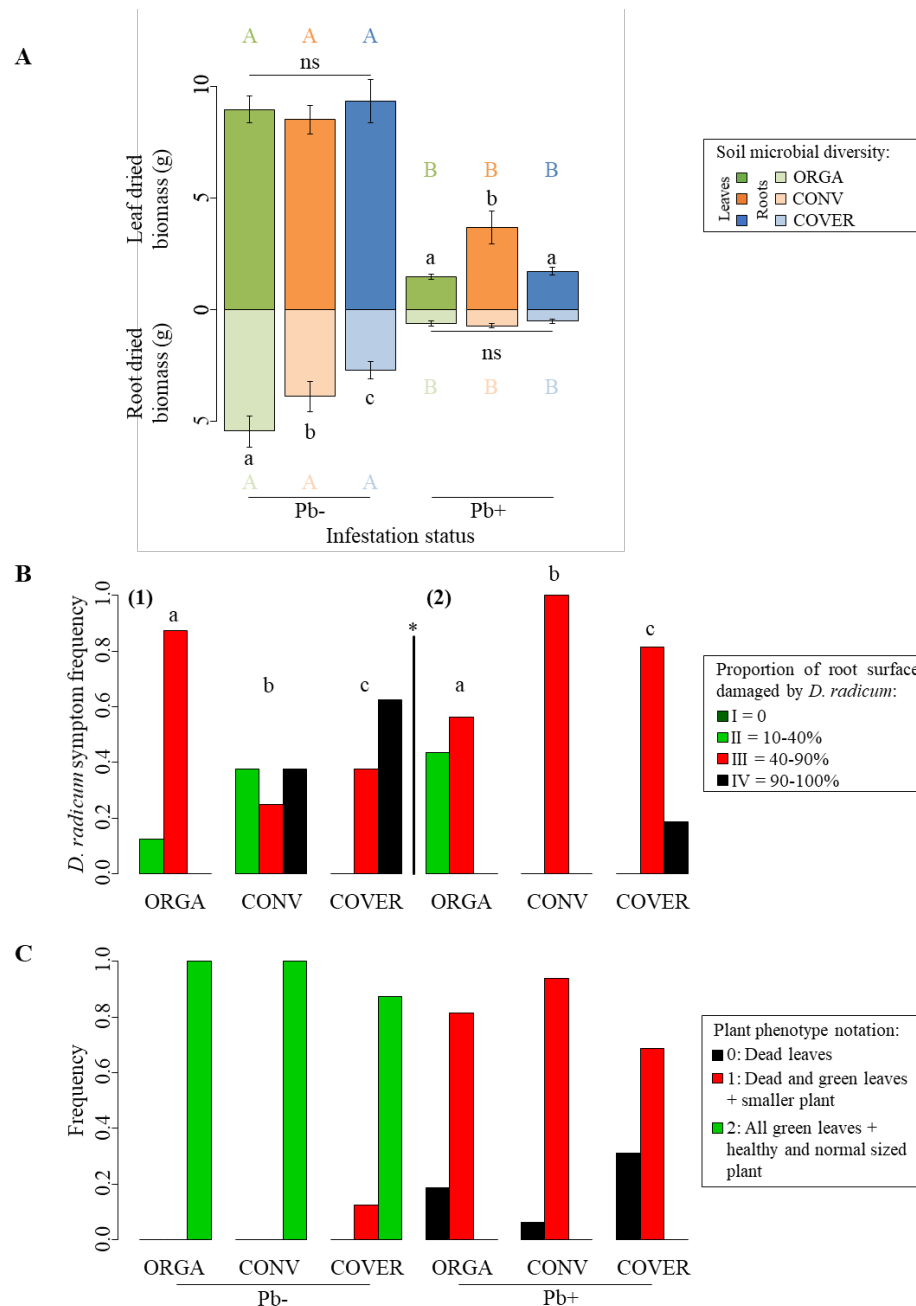


Figure 6 Plant growth and health modulated by the different soil microbial diversities. (A) Leaf and root mean (\pm se) biomasses, N = 16 plants per infestation status and soil microbial diversity. Lowercase letters indicate significant differences between soil microbial diversities for a given infestation status while uppercase indicate significant differences between infestation status for a given soil microbial diversity. (B) Root damages during the larval stage (1) and at the end of emergence (2), N = 8 and 16 plants per soil microbial diversity, respectively for the two sampling times. Lowercase letters indicate significant differences between soil microbial diversity for a given sampling time while the asterisk shows a difference between sampling time. (C) Overall plant phenotype assessment at the end of emergence, N = 16 plants per infestation status and soil microbial diversity. The “ORGA”, “CONV” and “COVER” diversities correspond to the organic, conventional and cover cropping agricultural practices while Pb- and Pb+ stand for without and with *P. brassicae*.

3.3. Fly phenotype

Emergence dynamics were globally impacted by the same factors in the two performed analyses, considering time either as a quantitative or as a qualitative variable (table 5). Initial soil microbial diversity, infestation status and time (i.e. period during which emergence was observed) significantly modified the fly emergence dynamics, as well as the interaction between infestation status and time, and soil replicate. In all soil conditions, the presence of Pb made the flies emerge earlier than the ones from the Pb- treatment (figure 7A). For Pb-, flies from the “ORGA” and “COVER” conditions emerged significantly earlier than the “CONV” condition. For Pb+, the three soil conditions had different emergence dynamics: flies from the “COVER” condition emerged the earliest while the ones from the “CONV” condition emerged the latest, and flies from the “ORGA” condition between both.

Emergence rate was significantly influenced infestation status and slightly by initial soil microbial diversity, but not by the interaction between both factors (table 5). Emergence rates were similar in the three soil conditions with or without Pb, while the presence of Pb decreased the emergence level in “ORGA” conditions (figure 7B). There was a higher variability of this trait in the Pb+ treatment (table 6).

Development time was significantly modulated by infestation status only, with the presence of Pb reducing the number of days required for the fly to complete its development (table 5, figure 7B). It ranged between 33 and 51 days for Pb- and between 33 and 45 days for Pb+ (table 6).

Survival time was significantly affected by the interaction between initial soil microbial diversity and infestation status, but the soil replicates were also significant (table 5). The presence of Pb increased the survival time in the “ORGA” condition (figure 7B). While survival time was similar in all three soil conditions for Pb-, it was at the highest in the “ORGA” condition and the lowest in the “COVER” condition for Pb+, with “CONV” as intermediate. The results of survival time was quite variable as it ranged from 0 to 11 days for Pb- and from 0 to 12 days for Pb+ (table 6).

Hind tibia length was significantly influenced by initial soil microbial diversity, infestation status and interaction between both factors (table 5). The presence of Pb increased tibia length for all soil conditions (figure 7B). For Pb-, the tibia length was not different between soil conditions but for Pb+, the pattern was similar to the survival time results, with the longest tibia for “ORGA” and the shortest for the “COVER”. Hind tibia length ranged from 1.54 to 2.14 mm for Pb- and from 1.50 to 2.26 mm for Pb+ (table 6).

Table 5 Outputs of the analyses of variance performed on each fly trait model. P values written in bold indicate a significant effect of the associated factor, also in bold.

Traits	Statistics
Emergence dynamics (time as a <u>quantitative</u> variable)	<p>Initial soil microbial diversity: Chisq=161.2; df=2; P<0.001</p> <p>Infestation status: Chisq=52.4; df=1; P<0.001</p> <p>Time: Chisq=3434.1; df=1; P<0.001</p> <p>Soil replicate: Chisq=84.9; df=3; P<0.001</p> <p>Initial soil microbial diversity:Infestation status: Chisq=2.6; df=2; P=0.26</p> <p>Initial soil microbial diversity:Time: Chisq=6.1; df=2; P=0.048</p> <p>Infestation status:Time: Chisq=213.9; df=1; P<0.001</p>
Emergence dynamics (time as a <u>qualitative</u> variable)	<p>Initial soil microbial diversity: Chisq=164.9; df=2; P<0.001</p> <p>Infestation status: Chisq=53.6; df=1; P<0.001</p> <p>Time: Chisq=4684.2; df=18; P<0.001</p> <p>Soil replicate: Chisq=87.2; df=3; P<0.001</p> <p>Initial soil microbial diversity:Infestation status: Chisq=1.9; df=2; P=0.38</p> <p>Initial soil microbial diversity:Time: Chisq=34.5; df=36; P=0.54</p> <p>Infestation status:Time: Chisq=397.0; df=18; P<0.001</p>
Emergence rate	<p>Initial soil microbial diversity: Chisq=6.21; df=2; P=0.044</p> <p>Infestation status: Chisq=5.05; df=1; P=0.024</p> <p>Initial soil microbial diversity:Infestation status: Chisq=0.91; df=2; P=0.63</p>
Development time	<p>Initial soil microbial diversity: Chisq=4.32; df=2; P=0.11</p> <p>Infestation status: Chisq=36.25; df=1; P<0.001</p> <p>Initial soil microbial diversity:Infestation status: Chisq=0.25; df=2; P=0.88</p>
Survival time	<p>Initial soil microbial diversity: Chisq=1.93; df=2; P=0.37</p> <p>Infestation status: Chisq=1.85; df=1; P=0.17</p> <p>Soil replicate: Chisq=7.01; df=3; P=0.07</p> <p>Initial soil microbial diversity:Infestation status: Chisq=10.5; df=2; P=0.005</p>
Hind tibia length	<p>Initial soil microbial diversity: F=6.72; df=2; P=0.034</p> <p>Infestation status: F=53.6; df=1; P<0.001</p> <p>Initial soil microbial diversity:Infestation status: F=6.15; df=2; P=0.046</p>

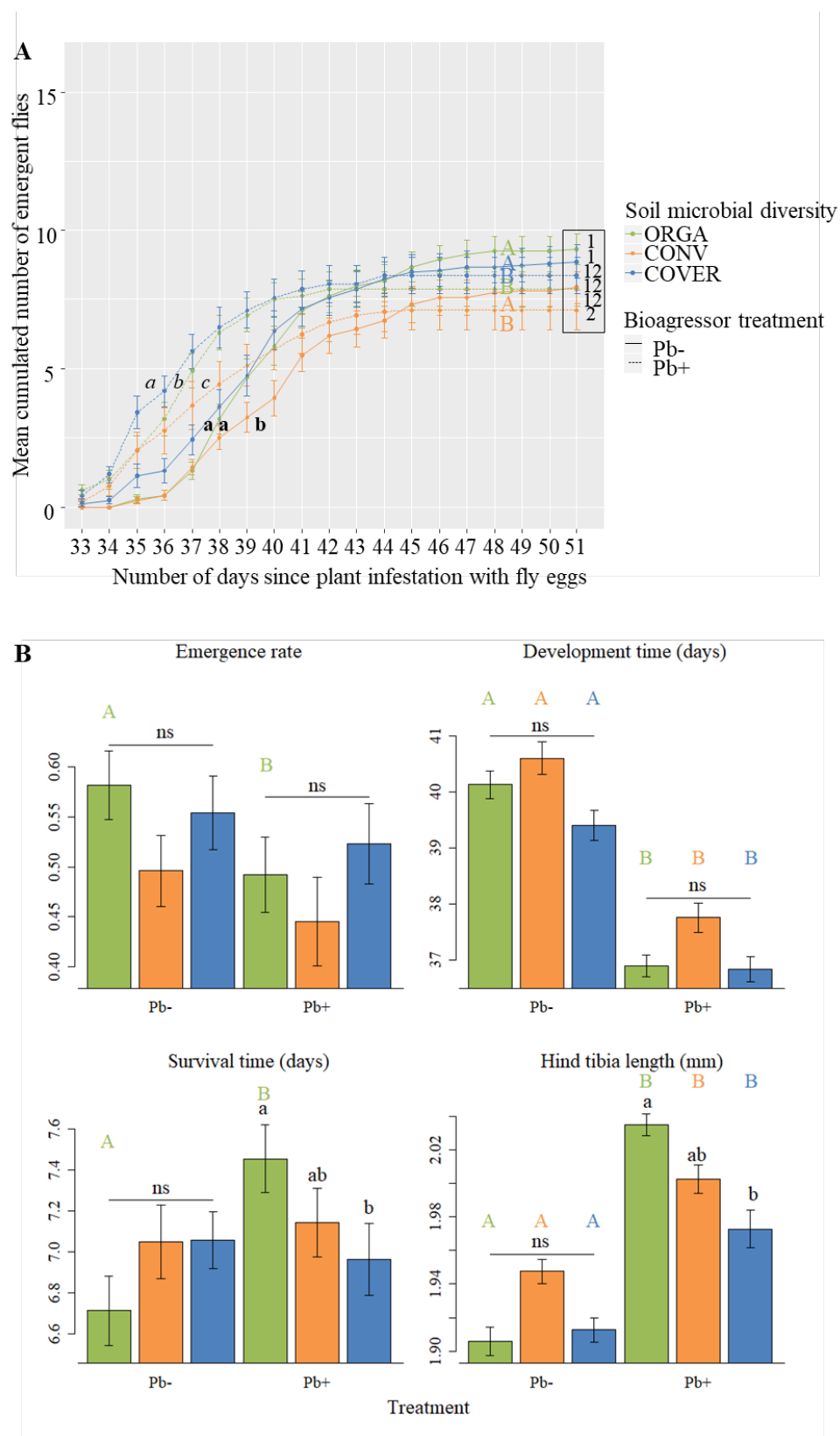


Figure 7 Life history traits of the CRF in presence and absence of *P. brassicae* (Pb+, Pb-). (A) Emergence dynamics and (B) emergence rate, development time, survival time and size (i.e. hind tibia length). The “ORGA”, “CONV” and “COVER” diversities (standing for organic, conventional and cover cropping agricultural practices) are respectively in green, orange and blue color. Lowercase letters indicate significant differences between soil microbial diversity for a given infestation status while uppercase indicate significant differences between infestation status for a given soil microbial diversity. On the plot of the emergence dynamics, the framed numbers indicate significant differences between the 6 curves at the final time.

Table 6 General statistics for each fly life history trait. “ORGA”, “CONV” and “COVER” stand for organic, conventional and cover cropping agricultural practices respectively, while “Pb-” and “Pb+” stand for without and with *P. brassicae*.

Infestation status	Initial soil microbial diversity (N flies)	Statistics	Emergence rate	Development time	Survival time	Size
Pb-	ORGA	Mean (se)	0.58 (0.034)	40 (0.244)	6 (0.169)	1.90 (0.008)
	N=149	Min/Median/Max	0.31/0.62/0.75	35/39/51	0/7/11	1.54/1.90/2.14
	CONV	Mean (se)	0.49 (0.035)	40 (0.295)	7 (0.180)	1.94 (0.007)
	N=127	Min/Median/Max	0.18/0.50/0.68	35/41/51	0/7/11	1.69/1.95/2.14
	COVER	Mean (se)	0.55 (0.036)	39 (0.272)	7 (0.138)	1.91 (0.007)
	N=142	Min/Median/Max	0.25/0.56/0.75	33/39/51	0/7/11	1.62/1.92/2.14
Pb+	ORGA	Mean (se)	0.49 (0.037)	36 (0.190)	7 (0.164)	2.03 (0.006)
	N=126	Min/Median/Max	0.18/0.50/0.81	33/37/42	0/8/12	1.79/2.04/2.26
	CONV	Mean (se)	0.44 (0.044)	37 (0.267)	7 (0.166)	2.00 (0.008)
	N=114	Min/Median/Max	0.18/0.50/0.81	33/37/45	0/7/11	1.73/2.01/2.26
	COVER	Mean (se)	0.52 (0.040)	36 (0.223)	6 (0.176)	1.97 (0.011)
	N=134	Min/Median/Max	0.25/0.53/0.75	33/36/44	1/7/11	1.50/2.00/2.22

Acknowledgements

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Short note 2

Diversity and association networks analysis of bacterial, fungal and viral communities associated to *Brassica napus* in a pathobiome context “BRASSICADIV-PATHO” project

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Key words: Microbial communities, networks, bioagresseurs, *Brassica napus*, pathobiome

1. Context and objectives

In the context of the holobiont concept, a plant can be seen as a dynamic unit, which microbiota plays a role in the plant growth and health under abiotic and biotic stresses (Bulgarelli *et al.*, 2012; Vandenkoornhuyse *et al.*, 2015). So far, rhizosphere and roots are the most studied habitat for microorganisms but recent studies have described other plant compartments like the phyllosphere (i.e. leaf) or the spermosphere (i.e. seeds) habitats (Barret *et al.*, 2015; Vacher *et al.*, 2016). Yet, few studies have an integrative view to describe the dynamics of several plant compartments. The work of de Souza *et al.* (2016) was the first to characterize the dynamics of bacterial and fungal communities rhizosphere, roots, caulosphere (i.e. stem habitat) and phyllosphere in mirror of sugarcane phenology. In the agricultural context, the influence of bioagressors on such dynamics remains to be unraveled.

Using oilseed rape (*Brassica napus*) as a crop plant, the “BRASSICADIV-PATHO” project aimed at i) characterizing the diversity, the composition and the interaction network structure of bacterial and fungal communities in the same habitat and between habitats (root, caulosphere, phyllosphere or spermosphere), ii) evaluating the dynamics of these communities xxx in mirror of *B. napus* phenology from sowing to harvest, and iii) assessing the effects of bioagressors on the root, caulosphere, phyllosphere and spermosphere microbial communities.

This project took place during the first year of my thesis and I was part of the people full-time involved for a year to carry out the experiment; organize and participate to the plant sampling; perform the various measurements that were done to characterize the plant growth and health; and analyze with statistics the obtained data.

2. Experimental design

The experiment took place at the Institute for Genetics, Environment and Plant Protection on the INRA site of Le Rheu (Brittany, France).

2.1. Implementation

A woven fabric of 2.10 m wide and 25 m long was displayed in a field, where were placed three lines of eight containers, with an interval of 2 m between each container and of 3 m between each line (figure 1A). Containers had a capacity of 600 L and were dimensioned as follows: 1,200 (length) x 1,000 (width) x 780 (height) mm, and pierced at two areas, at 10 cm

from the base for rainfall evacuation. For soil drainage, 8 cm of pozzolan were placed in each container with a geotextile layer on top. Then, the containers were filled with soil, as approximately 15 m³ of soil were collected in September 2016 from the layer 10-30 cm in the field of La Gruche (Pacé, France, N: 48°08.44', W: 01°47.98'), where wheat is usually cultivated and after crop harvest. Lastly, a 20 mm rain was simulated for each container over the following weeks, during which soil was being prepared for sowing, then watering naturally occurred when it rained.

The experimental site was equipped of a rain gauge, an anemometer, three sensors of air temperature and humidity while each container was equipped of a soil humidity sensor placed at 15 cm deep. All of these instruments were connected to a measurement and control datalogger (CR1000, Campbell Scientific). Air temperature and humidity, wind force, rain and soil humidity were recorded every hour throughout the duration of the experiment.

2.2. Sowing and application of bioagressor treatments

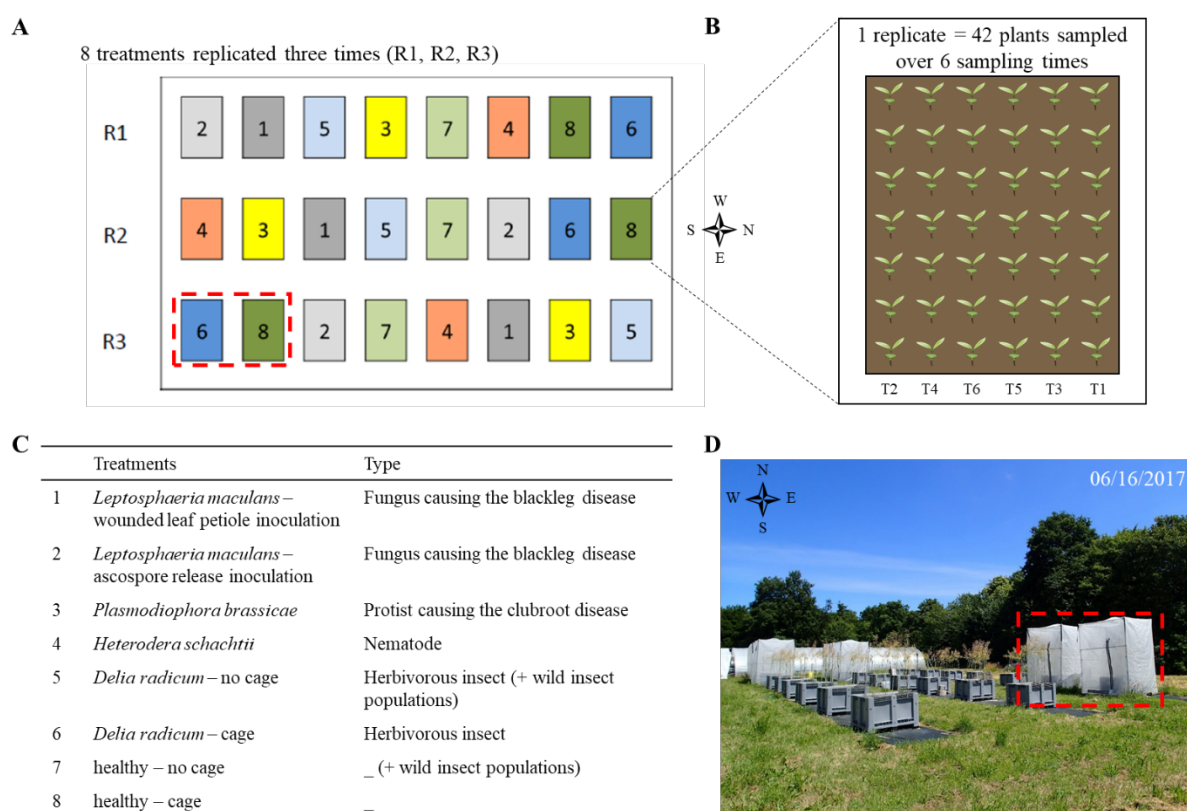


Figure 1 Schematic overview of the experimental site (A), one container with a view from above (B), the bioagressor treatments (C) and the implemented cages (D). Red dashed rectangle gives the location of the two same cages on the map of the experiment and on the picture.

The experimental setup was arranged in three blocks (i.e. R1, R2, R3) where treatments were randomly split (figure 1A). In each container, 42 seeds of *B. napus* L. (subsp. *oleifera* cv.

Bristol) were sown so that seven plants per container would be collected at each sampling time (from T1 to T6, figures 1B-2). Eight treatments of bioagressors were applied (figure 1C), among which were a fungus (with two ways of inoculation), a protist, a nematode, a rhizophagous insect (the cabbage root fly, *Delia radicum*) and a control (i.e. where plants remained healthy). The healthy and *Delia* treatments were doubled and half was subjected to the installation of cages before sowing to remove the effects of wild insect colonization (figure 1D).

Regarding the *Delia* treatment, the remaining 21 plants (i.e. T4, T5, T6) per container were infested with 15 eggs + 4 eggs (as hatching rate was of 75%) of *D. radicum* on the 11th of April, in spring. The population used in this experiment came from the laboratory where offspring of pupae, collected in the field in 2016 in Le Rheu (France), were reared on rutabaga roots in controlled conditions.

2.3. Plant sampling

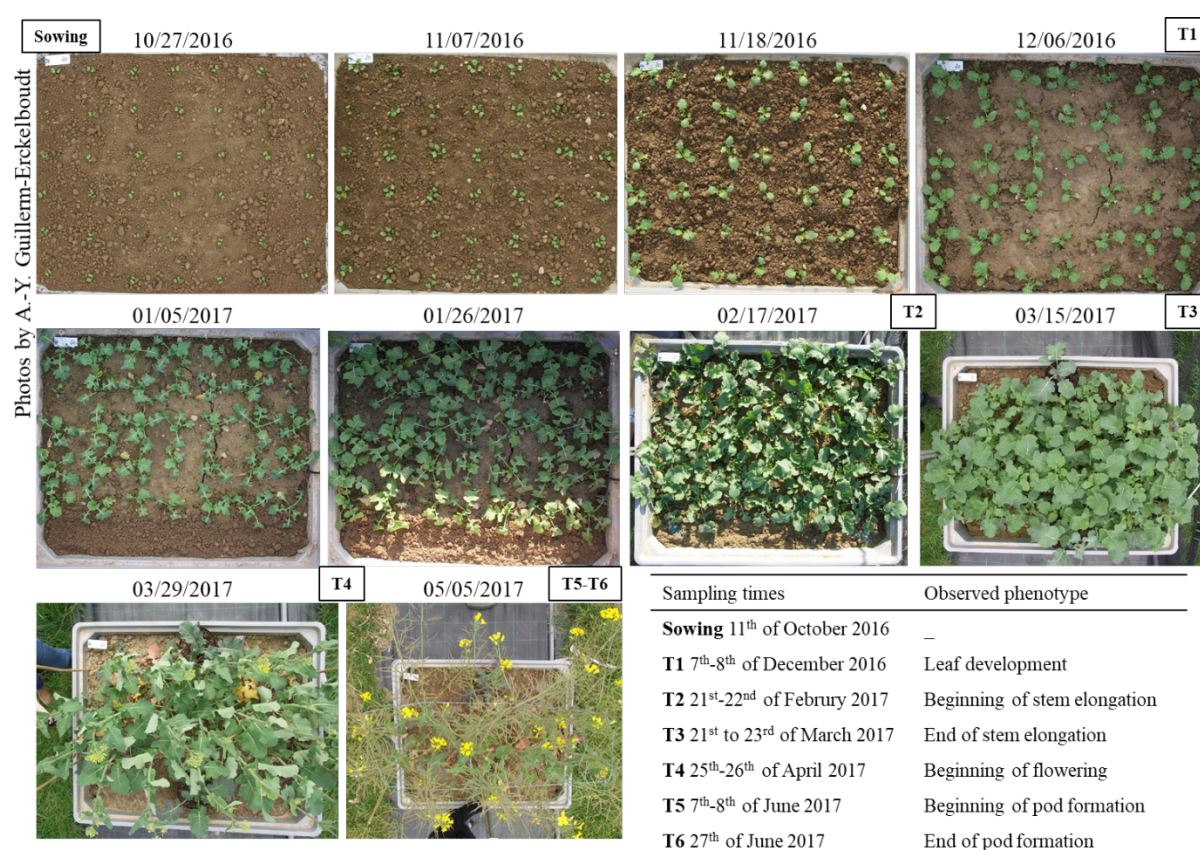


Figure 2 Sampling times distributed along the plant development. The container on the picture was from the healthy treatment without cage.

Six sampling were performed along the plant biological cycle (figure 2), during which plant rhizosphere, roots and leaves were collected. Stems were also sampled in healthy and *Leptosphaeria* treatments as the fungus ended its migration in the stem at some point of its

development. Seeds were also collected at the end of the experiment. These plant compartments were freeze-dried and stored at -20°C. Leaf, root and stem samples were grinded and aliquoted in plates, which were sent to the Gentyane platform (“Génotypage et séquençage en Auvergne”, Clermont-Ferrand, France) for DNA extraction.

2.4. Assessment of plant growth and health

While leaf and root biomasses were measured on samples collected at one of the six sampling times, several other traits were measured during the plant development to assess its growth and determine whether bioagressors could affect plant growth (table 1).

Table 1 List of the measurements realized during the experiment to assess the plant phenotype (i.e. growth and reproduction) and health (i.e. susceptibility to natural diseases and wild insect colonization).

Organism	Traits	Measurements
Plant	Growth	Leaf [#] and root dried biomasses – measured at every sampling time Height (i.e. stem elongation) – measured over several weeks Number of primary and secondary ramifications – counted at the end of stem elongation Length of primary ramifications – measured at the end of stem elongation
	Reproduction	Number of flowers – counted over several weeks Number of pods [#] – counted over several weeks and at T6 Number of seeds [#] – counted after the T6 1000-seed-weight [#]
Disease susceptibility	Assessed over several weeks on T6 plants and assessed on plants just before their sampling	<i>Leptosphaeria biglobosa</i> : number of maculae <i>Leptosphaeria maculans</i> : number of maculae <i>Pseudocercospora</i> : number of maculae <i>Alternaria</i> : number of maculae <i>Mycosphaerella</i> : number of maculae <i>Cylindrospori</i> ose: presence/absence and number of diseased leaves Powdery mildew: presence/absence <i>Sclerotinia sp</i> : presence/absence
	Assessed during sampling time	Length and notation of external necrosis caused by <i>Leptosphaeria maculans</i> Length and notation of internal necrosis caused by <i>Leptosphaeria maculans</i> Length of internal symptoms caused by <i>Leptosphaeria maculans</i>
Insect susceptibility during flowering and pod formation	Colonization [†] – counted over several weeks from mid-April to mid-June	<i>Meligethes sp</i> – number of individuals <i>Ceutorhynchus assimilis</i> – number of individuals <i>Ceutorhynchus napi</i> – stem damage measurement <i>Plutella xylostella</i> – number of individuals <i>Operophtera brumata</i> – number of individuals <i>Aphis sp</i> – number of individuals <i>Brevicoryne brassicae</i> – number of individuals <i>Myzus persicae</i> – number of individuals <i>Episyrphus balteatus</i> – number of individuals <i>Phyllotreta atra</i> – number of individuals <i>Psylliodes chrysocephala</i> – number of individuals
	Damages – assessed on plants just before their sampling	On the leaves: Lepidopterians and flea beetle On the stem: <i>Ceutorhynchus napi</i> On the pods: <i>Meligethes sp</i>

[#]At T6, distinction between the main stem and ramifications. [†]Wild insect colonization was not assessed in cages.

It is important to note that the experiment was realized in semi-field conditions, and therefore plants were subjected to natural disease infections and wild insect damage and colonization, which questions the impact of bioaggressor treatments on these events: would it increase or decrease disease and insect susceptibility?

Disease susceptibility and insect damages were assessed on plants before sampling (table 1). Also, disease susceptibility and wild insect colonization were followed over time respectively on the final row (i.e. plants sampled at T6) and the last three rows of plants (i.e. plants sampled at T4, T5 and T6).

3. Current situation and valorization

DNA extraction was performed on only half of the treatments (i.e. healthy, 2 *Leptosphaeria*, *Delia*) for the following reasons. Almost no gall was observed on the roots of the protist treatment, which would have indicated a successful development of the protest and no cyst was found back in the soil of the nematode treatment even several months after inoculation. The main reason for these treatments failing, is most probably due to the dry weather following protist and nematode inoculations, which normally require a certain level of soil humidity to develop. Lastly, most cages of both healthy and *Delia* treatments suffered high levels of aphid infestation.

However, infestation with *D. radicum* might have occurred too late to affect the plant phenotype: i) the plants were most probably too strong (i.e. massive taproot) though root damage was observed and ii) we can not exclude the fact that eggs underwent predation during this period as climate started to warm up. Therefore, only samples of healthy and *Leptosphaeria* treatments were sequenced since the fungus was the only bioaggressor to significantly affect the plant phenotype. Using Illumina MiSeq and different primer pairs (16S and *gyrB* or *rpoB* for bacteria, ITS for fungi), samples were sequenced at GeT-PlaGe platform (Toulouse, France) to assess bacterial and fungal communities in leaves, stems and roots. Bioinformatic analyses were achieved with MOTHUR and DADA2 pipelines. Ordination methods (i.e. tb-RDA) were performed on generated count data and networks were built with PLN-network method.

Currently, an article that will present *Leptosphaeria* vs healthy data, is being written and should be submitted before the end of the year. So far, fungal community structure has been described in the different plant compartments (i.e. roots, stems and leaves). The fungus *L. maculans* was integrated to the leaf community at the beginning of the experiment and migrated

to the stem and root communities. The dominant fungi that interacted with *L. maculans* in the different plant compartments were also identified. However, difficulties were encountered regarding the bacterial communities in the roots because of co-amplification with the plant. To overcome this problem, the perspectives would be i) that fungal communities could be focused on and bacterial communities momentarily given up; ii) to look for other primers that do not amplify the plant and *rpoB* could be a promising alternative (Ogier *et al.*, 2019). Moreover, building a network or several for each compartment, using PLN-network method (Poisson Log-normal) developed by Julien Chiquet from Agro Paris Tech, would permit to identify the key species that interact positively or negatively with *L. maculans* in the different plant compartments.

To go further, it would be relevant to isolate the identified key species from the networks in order to determine their functions and roles in plant-bioaggressor interactions in controlled conditions; and therefore to assess the plant chemical pathways (i.e. immunity, defense mechanisms) induced by the bioaggressor, as well as the key species involvement in the activation of these plant pathways. Identifications of key microorganisms and defense-induced pathways could be stepping-stones to the development of new tools for biological control.

SCIENTIFIC COMMITMENTS



SCIENTIFIC COMMITMENTS

1. Publications



Lebreton L., Guillerme-Erckelboudt A.-Y., Gazengel K., Linglin J., **Ourry M.**, Glory P., Sarniguet A., Daval S., Manzanares-Dauleux M. J. & Mougél C. (2019). Temporal dynamics of bacterial and fungal communities during the infection of *Brassica rapa* roots by the protist *Plasmodiophora brassicae*. PLoS ONE 14, e0204195. Doi:10.1371/journal.pone.0204195. **JIF₂₀₁₈ = 2.776**



Ourry M., Lebreton L., Chaminade V., Guillerme-Erckelboudt A.-Y., Hervé M., Linglin J., Marnet N., Ourry A., Paty C., Poinso D., Cortesero A.M. & Mougél C. (2018). Influence of belowground herbivory on the dynamics of root and rhizosphere microbial communities. Frontiers in Ecology and Evolution, 6:91. Doi: 10.3389/fevo.2018.00091. **JIF₂₀₁₈ = 2.686**



Lachaise T.*, **Ourry M.***, Lebreton L., Guillerme-Erckelboudt A.-Y., Linglin J., Paty C., Chaminade V., Marnet N., Aubert J., Poinso D., Cortesero A.M. & Mougél C. (2017). Can soil microbial diversity influence plant metabolites and life history traits of a rhizophagous insect? A demonstration in oilseed rape. Insect Science, 24(6): 1045-1056. Doi: 10.1111/1744-7917.12478. **JIF₂₀₁₈ = 2.710**

*Both authors contributed equally to this work.

2. Talks



Ourry M., Lebreton L., Chaminade V., Guillerme-Erckelboudt A.-Y., Hervé M., Linglin J., Marnet N., Ourry A., Paty C., Poinso D., Cortesero A.M. & Mougél C.. Deciphering *Brassica napus*-microbiome associations in interaction with root herbivorous insect *Delia radicum*: a feedback loop in the rhizosphere. **International Phytobiomes Conference 2018** hold on December 4th-6th 2018 in Montpellier (France).



Ourry M., Lachaise T., Lebreton L., Guillerme-Erckelboudt A.-Y., Linglin J., Paty C., Chaminade V., Marnet N., Aubert J., Poinso D., Cortesero A.M. & Mougél C.. Can soil microbial diversity influence plant metabolites and life history traits of a rhizophagous insect? **International conference on Ecological Science (SFÉcologie 2018)** hold on the October 22nd-25th 2018 in Rennes (France).



Ourry M., Lebreton L., Chaminade V., Guillerm-Erckelboudt A.-Y., Hervé M., Linglin J., Marnet N., Ourry A., Paty C., Poinot D., Cortesero A.M. & Mougél C.. Influence of belowground herbivory on the dynamics of root and rhizosphere microbial communities. **International conference of Brassica 2018** hold on July 2nd-4th 2018 in Saint Malo (France) and National conference organized by and for the PhD students from the “**Plant Healthy and Environment**” department of the French National Institute for agricultural research hold on June 27th-29th 2018 in Nice (France).

3. Posters



Ourry M., Lebreton L., Chaminade V., Guillerm-Erckelboudt A.-Y., Hervé M., Linglin J., Marnet N., Ourry A., Paty C., Poinot D., Cortesero A.M. & Mougél C.. Deciphering *Brassica napus*-microbiome associations in interaction with root herbivorous insect *Delia radicum*: a feedback loop in the rhizosphere. Poster presented at the **First Scientific Meeting of the EGAAL Doctoral School** hold on July 4th and 5th 2019 in Rennes, at the National conference organized by and for the PhD students from the “**Plant Healthy and Environment**” department of the French National Institute for agricultural research hold on May 21st-23rd 2019 in Rennes (France) and at the **International Phytobiomes Conference 2018** hold on December 4th-6th 2018 in Montpellier (France).



Gravot A., Hamzaoui O., Lebreton L., Marnet N., Marquer B., Leconte P., Langrume C., Lariagon C., Lemoine J., Lode M., Berardocco S., Gilet M.-M., Trotoux G., Glory P., Domin C., Moulin B., **Ourry M.**, Mougél C., Laperche A., Nesi N., Chèvre A.-M., Rousseau-Gueutin M., Cortesero A.M., Hervé M., Manzanares-Dauleux M. J., Delourme R. & Bouchereau A. (2018). ‘DEsCriBe’ project: A wide investigation of phytochemical diversity in leaves, roots and seeds of *Brassica napus*. Poster presented at the **International Conference of Brassica 2018** hold on July 2nd-4th 2018 in Saint Malo (France).



Lebreton L., Chaminade V., Cortesero A.-M., Daval S., Fournet S., Gazengel K., Guillerm-Erckelboudt A.-Y., Lachaise T., Linglin J., Manzanares-Dauleux M., Montarry J., **Ourry M.**, Paty C., Poinot D., Porte C. & Mougél C. (2018). Does the soil microbiota diversity influence the interactions between *Brassica napus* and its bioaggressors? Poster presented at the **International Conference of Pathobiome** hold on March 18th-20th 2018 in Ajaccio (Corsica, France).



Ourry M., Cortesero A.M. & Mougél C. (2017). Interaction between soil microorganisms, plant and insect. Poster presented during the “**Plant microbiota**” practical course organized by the **European Molecular Biology Organization** from March 27th to April 7th 2018 at the “Max Planck Institut für Pflanzenzüchtungsforschung” in Cologne (Germany).

4. Awards

INRA Early Career Prize: The INRA PhytoMic network, supported by the Microbial Ecosystems and Meta-omics Metaprogram, wished to encourage the participation of early career scientists to the International Phytobiomes Conference 2018 through the funding of eight awards of 500 € each.

DECIPHERING *BRASSICA NAPUS*-MICROBIOME ASSOCIATIONS IN INTERACTION WITH ROOT HERBIVOROUS INSECT *DELIA RADICUM*: A FEEDBACK LOOP IN THE RHIZOSPHERE



OURRY M.¹, LEBRETON L.¹, CHAMINADEV.¹, GUILLERM-ERCKELBOUDT A.Y.¹, HERVÉ M.¹, LINGLIN J.¹, MARNET N.^{1,2}, OURRY A.³, PATY C.¹, POINSOT D.¹, CORTESERO A.M.¹, MOUGEL C.¹

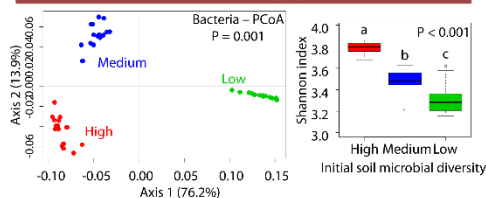
CONTEXT AND STUDY OBJECTIVES

Plants are known to be major biological hubs, which above- and belowground organs interact with either beneficial or detrimental macro- and microorganisms. Even though plant-microorganism interactions are better understood, tripartite interactions involving herbivorous insects remain more complex. By studying the feedback loop of the tripartite interaction between soil microorganisms, a crop plant and a belowground herbivore, the aims were i) to assess the effects of the interaction between the plant and different soil microbial communities on the herbivore development and ii) to determine in return how root chemistry modulated by herbivory could modify the root and rhizosphere microbiota.

MATERIALS AND METHODS

Soils of different initial microbial diversities (high, medium, low) obtained through the dilution to extinction method were used. Leaf, root and rhizosphere samples were collected according to major development stages of the herbivore: hatching, larva, emergence. Plant primary (amino acids, sugars) and secondary (glucosinolates) metabolites and chemical elements were quantified while bacterial and fungal community assemblages of roots and rhizosphere were analyzed using a metabarcoding approach (16S and 18S rDNA genes); and the insect life history traits (oviposition, emergence rate and fitness) were measured.

Soil manipulation enabled the realization of a microbial diversity gradient



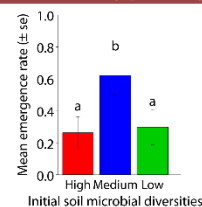
Three soils of different bacterial structure (β -diversity) and Shannon index (α -diversity) were obtained by using the dilution to extinction method. Similar results were observed for fungi.

Root metabolites varied according to the infestation dynamics

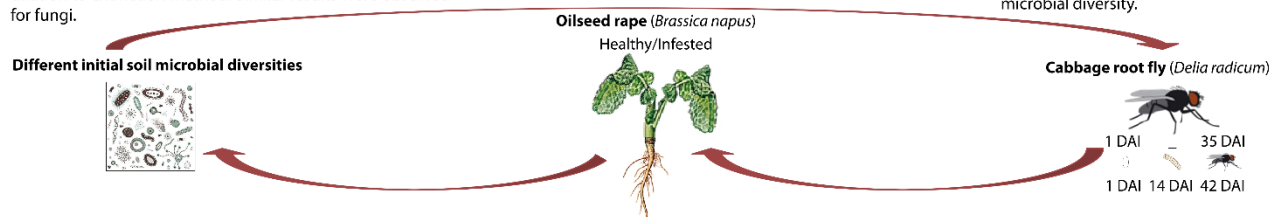
Factors	F value	df	P value
Initial soil microbial diversity	1.16	2	0.299
Treatment	14.30	2	0.001
Interaction	0.65	4	0.899

Initial soil microbial diversity did not influence the plant chemistry, which did not explain the observed change in the fly phenotype. However, infestation dynamics (i.e. treatment) seemed to induce major chemical changes.

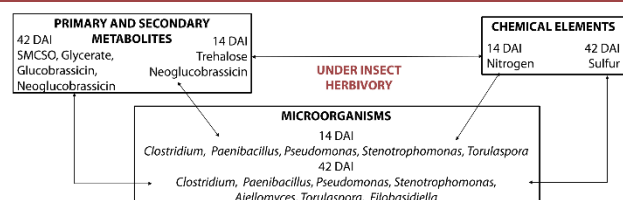
Microbial diversity influenced the fly phenotype



Emergence rate was at the highest on plants grown on medium soil microbial diversity.

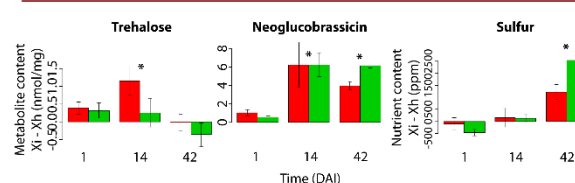


Chemical data can be associated to microbiota data under herbivory



The increase observed for some metabolites and chemical elements can be associated to an enhancement of several microbial genera during the peak or at the end of herbivory.

Herbivory increased several chemical compounds



Root herbivory induced a decrease of most chemical compounds but an increase of certain sulfured compounds (glucosinolates such as glucobrassicin and neoglucobrassicin) and sugars (trehalose, glycerate).

POTENTIAL SCENARIO Glucosinolates (e.g. neoglucobrassicin) are toxic sulfured compounds, which production increases under insect herbivory (van Dam & Raaijmakers, 2006). Trehalose is a sugar with a potential role in plant defenses, controlling herbivore abundance (Singh *et al.*, 2011). It can be either produced by *Stenotrophomonas* or consumed by *Pseudomonas* (Wolf, 2002; De La Fuente *et al.*, 2007). Oilseed rape needs high level of sulfur, and some *Pseudomonas* strains might have the capacity to produce an arylsulfatase enzyme, which promotes sulfur mineralization and a better access to sulfur for the plant (Knauff *et al.*, 2003; Gregut *et al.*, 2009). The new available sulfur could be used by the plant to continue the production of glucosinolates.

CONCLUSIONS AND PERSPECTIVES

Through a dilution to extinction manipulation, we have shown that diversity of soil microorganisms influenced the fly development that could not be explained by primary and secondary metabolites, strongly modulated by infestation dynamics. In return, we confirmed that belowground herbivory led to root chemical changes, involving mostly sulfur-containing compounds, and to shaping belowground microbial communities. It indicated that a plant suffering from herbivory emits either defensive and/or nutritive compounds that influence the recruitment of soil microorganisms by the rhizosphere and the roots.

Our results encourage the determination of the precise identity and functions of microorganisms responding to herbivory, using more accurate primers and metatranscriptomic approaches, and the understanding of the feedback-loop existing between these identified microorganisms and the plant chemistry modified by herbivory. This future research could represent the next step toward the transition from correlation to causation in order to develop sustainable and innovative plant protection strategies.

REFERENCES

Lachaise, Ourry *et al.* (2017). Can soil microbial diversity influence plant metabolites and life history traits of a rhizophagous insect? A demonstration in oilseed rape. *Insect Sci.* 24, 1045–1056. doi:10.1111/1744-7917.12478. Ourry *et al.* (2018). Influence of Belowground Herbivory on the Dynamics of Root and Rhizosphere Microbial Communities. *Front. Ecol. Evol.* 6. doi:10.3389/fevo.2018.00091.

'DEsCriBe' project: A wide investigation of phytochemical diversity in leaves, roots and seeds of *Brassica napus*

Antoine Grivot¹, Oumayma Hamzaoui¹, Lionel Lebreton¹, Nathalie Marnet², Bruno Marquer¹, Patrick Leconte¹, Christophe Langrume¹, Christine Lariagon¹, Jocelyne Lemoine¹, Maryse Lode¹, Solenne Berardocco¹, Marie-Madeleine Gilet¹, Gwenn Trotoux¹, Pascal Glory¹, Claude Domin¹, Bernard Moulin¹, Morgane Ourry¹, Christophe Mougell¹, Anne Laperche¹, Nathalie Nesi¹, Anne-Marie Chèvre¹, Mathieu Rousseau-Gueutin¹, Anne-Marie Cortesero¹, Maxime Hervé¹, Maria J. Manzaneres-Dauleux¹, Régine Delourme¹, Alain Bouchereau¹

Project Overview

Intraspecific phytochemical diversity is a key determinant of crop biotic interactions and source of plant resistance traits. Breeding efforts for low glucosinolate (GSL) contents in seeds have largely shaped the phytochemical landscape of oilseed rape varieties. The genetic architecture of GSL profile in seeds and leaves has been investigated by many research groups. However, several aspects of the phytochemical intraspecific diversity in vegetative parts of *Brassica napus* still deserve further extensive investigations. DEsCriBe is a recent project IGEPP, aiming at broad spectrum profiling of specialized compounds in a diversity panel of 304 accessions including low and high GSL winter and spring oilseed rape varieties, forage rape, swedes, and core collections of the two progenitor species *B. oleracea* and *B. rapa*. The first part of this work has just started, and focuses on endogenous (seeds, roots and leaves) secondary compounds, with a specific emphasis on glucosinolates and phenolics. Current concerns include the controlled and reproducible production of root and leaf samples for a large number of genotypes, and the development of analytical procedures dedicated to the assessment of putatively rare compounds that might be specific of some genotype subsets.

A 304-accession panel to investigate the phytochemical diversity in *B. napus*

Table 1: Plant accessions used in this work.

Eruic acid / Glucosinolates content in the seeds	Number of accessions				
	total	++	0/+	+/0	0/0
Winter oilseed rape accessions (<i>B. napus</i>)	130	41	18	1	70
Spring oilseed rape accessions (<i>B. napus</i>)	120	58	13		49
Swede accessions (<i>B. napus</i>)	10				
Forage rape accessions (<i>B. napus</i>)	10	8	1		1
Recent winter rapeseed varieties	11				11
<i>B. rapa</i> accessions	12				
<i>B. oleracea</i> accessions	11				

Cultivated *B. napus* varieties divide into winter and spring oilseed rape, swede and forage rape accessions. 21st-century breeding efforts led to the diminution of erucic acid and/or glucosinolates (GSL) in seeds. To maximize the overview of phytochemical diversity in rapeseed, the panel of varieties used in the DEsCriBe project includes several 'old' and 'recent' accessions from these different sub-groups. *B. napus* is an allopolyploid species derived from very few post-neolithic natural crosses between *B. rapa* and *B. oleracea*. To explore the possible bottleneck exerted by this evolutionary event on the phytochemical diversity of *B. napus*, we included accessions representing the genetic diversity of each of these two parental species in the panel.

Plant culture experimental design

Minimizing the influence of environmental variations was a major objective for such a large accession panel. In the first part of the study the work was focused on a single sampling time (4 weeks after sowing), with non-stressed plants and only two organs (roots and leaves).

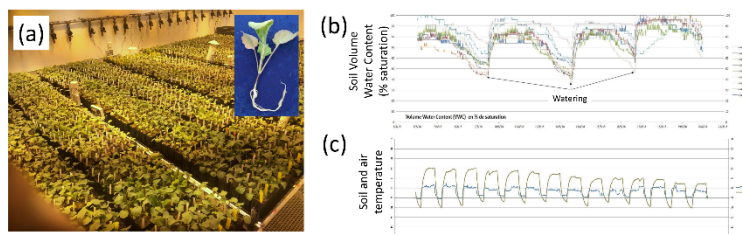


Figure 1: Experimental design for the production of plant samples

(a) View of the phytotron-cultured 304 plant genotypes (Photoperiod 16/8). Seeds were sown in a mix of soil and sand (2:1). Fertilization was applied twice with Hoagland ½ (eq. to 9 mg of inorganic nitrogen before sowing, and additional 9 mg two weeks after sowing). To make possible further studies on the connections between root metabolome and rhizosphere microbiome, culture conditions were chosen in such a way that the microbiological release of soil organic nitrogen content could play a significant role in plant nutrition. This is one of the three biological replicates (scaled over three successive months of culture). For each replicate, 7-8 plant individuals (displayed in two blocks) are cultivated for each genotype. Measurement of micro-climatic variables was performed with 6 "Watchdog 1400" recorders (Spectrum Technologies), equipped with 20 capacitive soil moisture sensors (Waterscount SM100), and 4 soil temperature sensors (3667-20). In addition, 6 air-temperature recorders (iButton Maxim Integrated) were displayed in the phytotron. (b) Plant watering was triggered by the continuous measurement of soil volume water content, in a way that maintained values between 100% and 40%. (c) Temperature setting of the phytotron device was driven by the measurement of soil temperature (5 cm below soil surface).

Setting of analytical procedures for targeted analyses of phenolics and glucosinolates



Preliminary work consists in the constitution of a qualitative chemical library of phenolics and glucosinolates in the tissues of *Brassica* accessions. Representative samples from the genetic panel are analyzed using combinations of approaches including 1/ mass fragmentation of every UV-absorbing compounds (Fig. 2) and systematic parent scan of compounds releasing GSL-fragments and 2/ HR-mass based metabolic screening. This work is currently developed using a column CSH C18 (2.5 µm). This column chemistry provides a satisfying retention of phenolics and of the most polar glucosinolates. This granulometry allows an utilization with both the LTQ-Orbitrap (for structural explorations), and TQD (for subsequent routine quantitative profiling of the full *B. napus* panel).

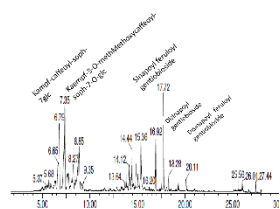


Figure 2: Example of major phenolic compounds identified in a leaf sample of a swede variety

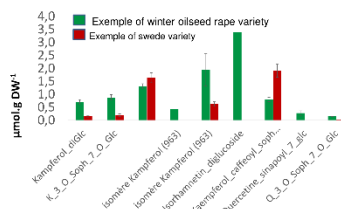


Figure 3: Illustration of inter-variety variations in leaf flavonol content. The absence of isorhamnetin derivatives in this swede variety may reflect a genetic defect in the production of the dedicated O-methyltransferase

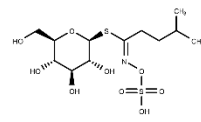


Figure 4: Structure of a ramified aliphatic glucosinolate.

Ramified and benzyl-conjugated glucosinolates have been rarely reported in *Brassica* species. Our preliminary work suggests their putative presence in some of our accessions, thus requiring additional structural validation

Perspectives

As main outputs, the DEsCriBe project will

- Define a **phytochemical identity card for every *B. napus* accession** in the panel, a dataset that will support the choice of genotypes in further works on plant biotic interactions.
- This study will improve our understanding of the **impact of recent *B. napus* breeding history on the metabolomics features of vegetative organs**.
- The dataset will allow a Genome Wide Association Study (GWAS) of **phytochemical diversity in *B. napus***, that may give starting points for further investigations on the molecular basis of the biosynthesis and regulation of rare or understudied *Brassica*-specific specialized metabolites that are absent from Arabidopsis.
- This work is connected to the project MAGNUM, where microbiome sequencing-based analysis of the root samples will give indications on the **relationships between plant genetics / metabolome and root microbial ecology**

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²Plateau de Profilage Métabolique et Métabolomique (P2M2), Centre de Recherche Angers Nantes BIA, INRA, Le Rheu, France.

Financial support: PROMOSOL

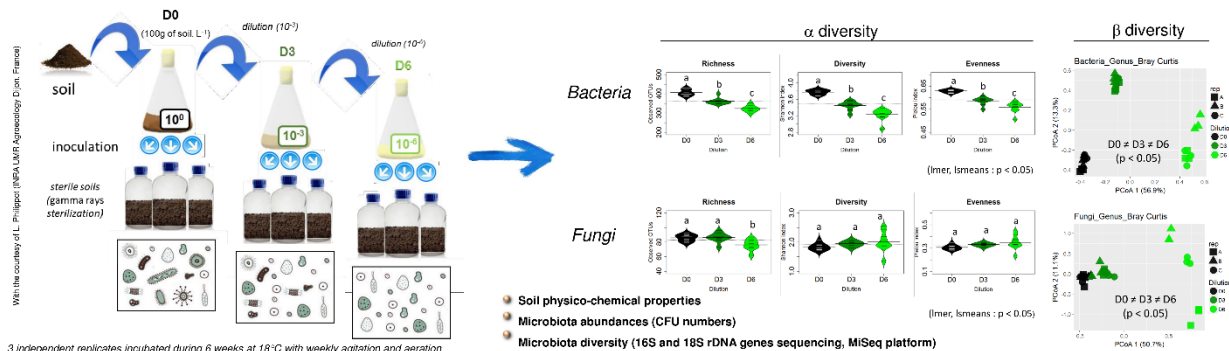
Does the soil microbiota diversity influence the interactions between *Brassica napus* and its bioaggressors ?

LEBRETON L., CHAMINADE V., CORTESERO A.M., DAVAL S., FOURNET S., GAZENGEL K., GUILLERM-ERCKELBOUDT A.Y., LACHAISE T., LINGLIN J., MANZANARES-DAULEUX M., MONTARRY J., OURRY M., PATY C., POINSOT D., PORTE C. AND MOUGEL C.

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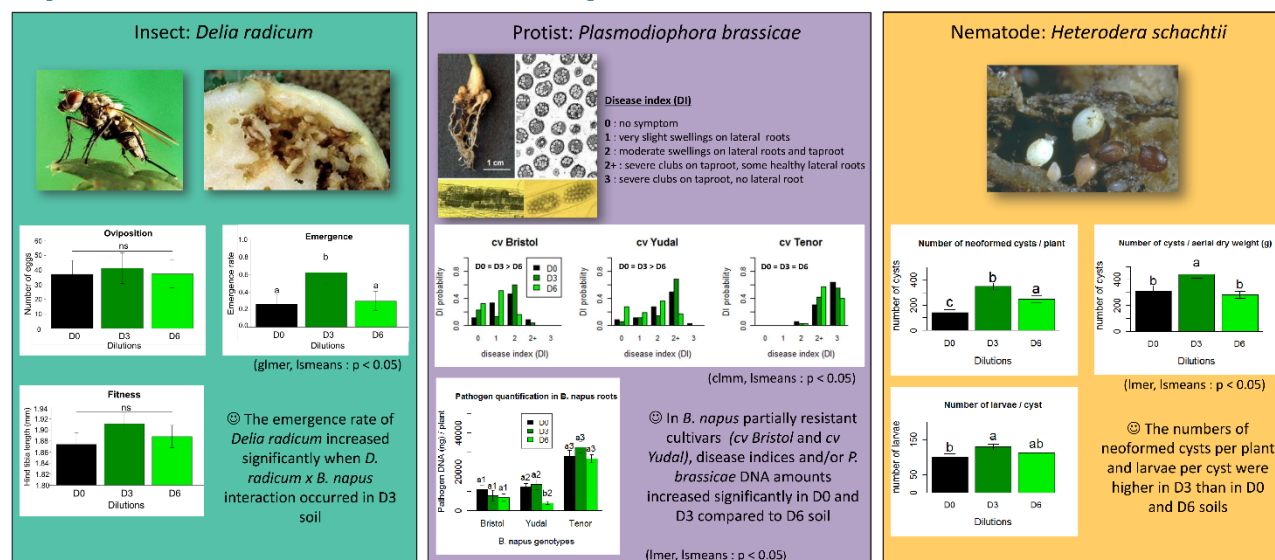
An overall perspective of the holobiont concept is to analyse the consequences of plant * microbiota interactions on plant defenses and bioaggressor traits linked to their fitness and parasitism. For this purpose, three soil preparations with different microbial diversities but with the same abiotic properties were obtained from one native soil by a removal/recolonization method. After interaction experiments carried out under controlled conditions between *B. napus* and three of its bioaggressors in these three soils, bioaggressor traits were compared. The initial diversity of soil microbial communities influences the traits of bioaggressors in interaction with *Brassica napus*.

1. EXPERIMENTAL MANIPULATION OF MICROBIAL DIVERSITY, *i.e.* THE SAME ABIOTIC ENVIRONMENT WITH DIFFERENT DIVERSITIES OF MICROBIAL COMMUNITIES



Three soils with three initial microbial richness and diversity levels were obtained through a removal/recolonization method
A better dilution effect on diversity parameters for bacteria than fungi communities

2. [BRASSICA NAPUS * SOIL MICROBIOTA] * BIOAGGRESSORS INTERACTIONS: KEY RESULTS



The fitness of bioaggressors increased when the interactions were carried out in soils of intermediate diversity compared to the soils of weak and strong diversity

Modification in the soil microbial diversity was associated with modification in plant metabolite profiles (Lachaise *et al.*, 2017)

Our results highlighted the holobiont concept and we need now a broader view of the link between diversity and function of microbial communities modulating traits related to plant defenses against bioaggressors in relation with the genetics of plant

INTERACTION BETWEEN SOIL MICROORGANISMS, PLANT AND INSECT

MORGANE OURRY⁽¹⁾, ANNE-MARIE CORTESERO⁽²⁾, CHRISTOPHE MOUGEL⁽¹⁾

Scientific context

It is common knowledge that insect herbivory activates the plant defence system. Indirect defences include emission of volatile organic compounds which attract parasitoids or predators while direct defences correspond to secondary metabolites with either repulsive or toxic effect. Several studies have also shown that the interaction between plants and soil microorganisms bring benefits to the plant and generated a new concept called "holobiont" (Figure 1). This characterizes a eucaryota host and its microbial communities as being a new entity which is influenced by its hologenome, corresponding to the host genome and the metagenome of the microbial communities. Little is known about interactions between soil microorganisms, plants and insects, the influence of pest on soil microbiota and vice versa, as well as the impact on plant defences and insect life history traits.

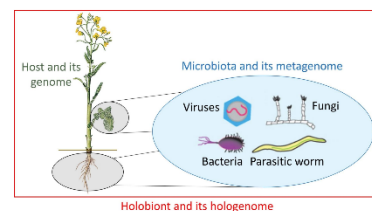
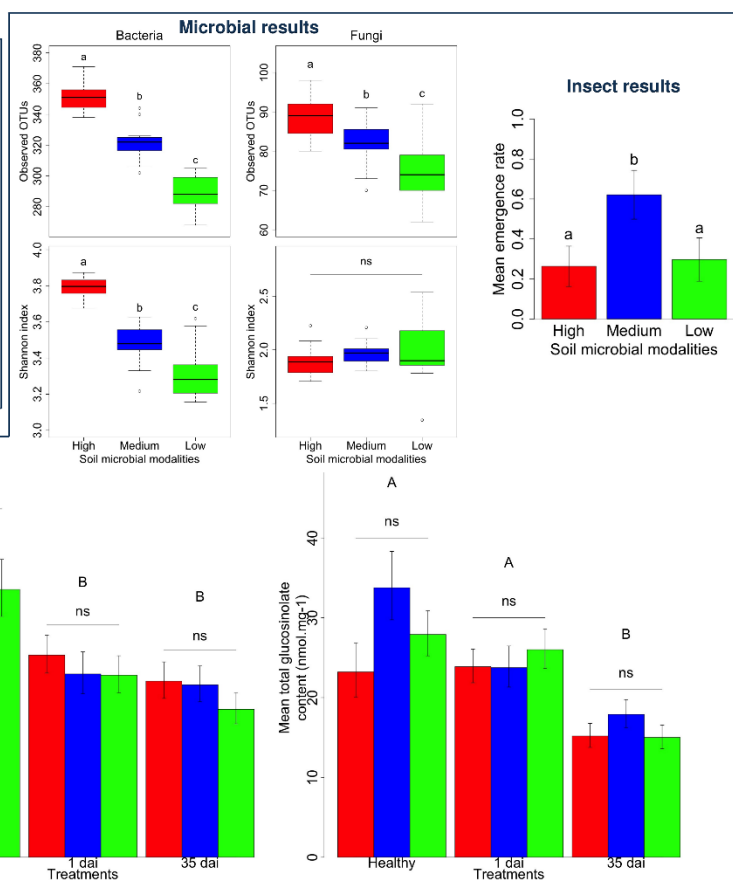
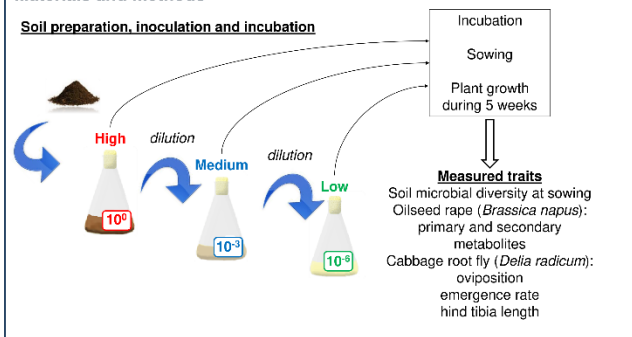


Figure 1: Schematic illustration of the concept of holobiont.

Past researches: Can soil microbial diversity influence plant metabolites and life history traits of a rhizophagous insect? A demonstration in oilseed rape. (Lachaise T.*, Ourry M.* *et al.*, 2017)

Materials and methods

Soil preparation, inoculation and incubation



Future researches

During my PhD work, I will attempt to answer the following questions:

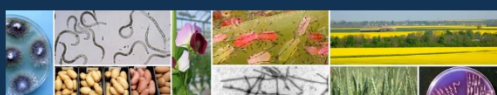
- How infestation by *D. radicum* influence the spatio-temporal dynamics of oilseed rape (*B. napus*) microbial communities in the rhizosphere and the phyllosphere (Figure 2)?
- Does infestation by *D. radicum* impact colonization by wild aboveground insect populations? What is the relation with plant microbial communities (Figure 2)?
- Can specific soil entomopathogenic species affect *D. radicum* oviposition, development and survival? Is the effect direct or indirect (i.e. through modification of plant defences)?

Perspectives

This PhD would be a preamble to researches focusing on the plant capacity to select microbial species and genes to promote its defences, hence resistance against insect pests.



Figure 2: Experimental design used to answer questions i and ii. Plant roots and leaves are sampled at several times, corresponding to different development stages of oilseed rape. Question i requires to use cages when infesting plants by *D. radicum* while question ii does not in order to follow wild insect populations.



FRENCH SUMMARY/RESUME EN FRANÇAIS

Chapitre I – Introduction générale

1. Cadre conceptuel

La symbiose est une relation intime entre deux organismes vivants appartenant à des espèces différentes, et ce terme est fréquemment utilisé dans le cadre d'interactions mutualistes. Les interactions symbiotiques peuvent se produire entre des organismes de règnes et/ou de tailles différents, à des échelles ou dans des écosystèmes variés. La symbiose peut induire des modifications morphologiques, physiologiques ou comportementales sur les organismes impliqués dans la symbiose, voire entraîner des changements dans leurs processus génétiques et évolutifs. Ces modifications ont conduit au nouveau concept de phénotype étendu, développé par Dawkins en 1982, où le phénotype observé est la résultante des gènes de l'hôte mais également d'autres organismes, tels que les microorganismes.

Le concept de symbiose a ensuite évolué vers le concept d'holobionte. D'après Lynn Margulis en 1993, un holobionte correspond à une unité biologique complexe à l'intérieur de laquelle interagissent un macroorganisme, appelé l'hôte, et des microorganismes, appelés symbiotes. Ce concept est maintenant fréquemment utilisé et les organismes tels que l'homme, les plantes ou les insectes sont connus pour héberger un panel de symbiotes, et plus largement des communautés microbiennes ou microbiote. Celles-ci rassemblent des microorganismes de règnes différents (ex. bactéries, champignons...) qui interagissent entre eux dans un environnement défini. De plus, ces communautés microbiennes vivant au sein d'une plante ou d'un animal peuvent être transmises verticalement, des parents aux descendants, ou horizontalement, de l'environnement ou d'autres organismes. Elles sont aussi très dynamiques, avec une composition microbienne variant au cours du développement de leur hôte et selon des facteurs biotiques ou abiotiques.

2. Interactions plantes-insectes dans le concept “holobionte” - comment et dans quelle mesure ces interactions peuvent-elles être modulées par des microorganismes?

Les insectes phytophages se nourrissent de divers tissus de la plante, que ce soit les parties aériennes ou souterraines. Ils peuvent se différencier selon leur méthode pour se nourrir (ex. piqueur-suceur ou broyeur) ou de spécialisation à l'hôte (ex. généraliste ou spécialiste).

Pour lutter contre les bio-agresseurs, les plantes mettent en place des mécanismes de défenses, soit constitutives et en permanence maintenues, soit induites par l'attaque d'insectes phytophages. Dans le cas de défenses directes, la plante va mettre en place des défenses physiques empêchant l'insecte de se nourrir, ou émettre des composés chimiques pouvant repousser ou être toxiques pour l'insecte. Par exemple, les glucosinolates sont des composés soufrés, principalement produits par des plantes de la famille des Brassicacées, qui peuvent avoir un effet négatif sur le développement des insectes phytophages. Les plantes peuvent aussi émettre des défenses indirectes qui ont pour but d'attirer les ennemis naturels (ex. prédateurs ou parasitoïdes) des insectes phytophages, par exemple via le déploiement de ressources ou d'abris, ou par l'émission de composés organiques volatiles (COVs) par la plante suite à une attaque. De plus, les défenses des plantes peuvent être induites par l'activation des voies de signalisation associées aux phytohormones.

Les plantes vivent en interaction avec leur environnement et d'autres organismes comme des microorganismes, structurés en communautés. Le compartiment racinaire de la plante est très étudié pour ses interactions avec les microorganismes. Les nodules et les mycorhizes sont deux exemples de symbioses entre les racines et des microorganismes (bactérie et champignon respectivement), qui améliorent la nutrition et donc la croissance de la plante. La rhizosphère, région du sol directement en contact et sous influence des racines, est également un compartiment très étudié. Cette zone très dynamique est un lieu d'échanges entre la plante et le sol dans laquelle habite une variété de microorganismes (ex. nématodes, arthropodes, virus, champignons, bactéries...). Les communautés microbiennes associées à la plante peuvent provenir de différents inocula, tels que la graine qui permet une transmission verticale du microbiote, ou le sol, l'air ou d'autres organismes interagissant avec la plante pour une transmission horizontale. Dans le cas de la rhizosphère, le sol constitue un réservoir important de diversité biologique qui dépend notamment de ses propriétés physico-chimiques, de sa géographie, de son couvert végétal, des pratiques culturales et du climat. La plante produit des composés carbonés (ex. exsudats racinaires, COVs) servant de nutriments pour les microorganismes et ainsi contribuant au recrutement des microorganismes du sol qui vont permettre une meilleure nutrition et croissance de la plante, et à sa réponse aux stress, ou une meilleure protection face à des bioagresseurs par exemple.

Les insectes vivent également en interaction avec des microorganismes, aussi souvent appelés symbiotes. Certains sont considérés comme obligatoires si les insectes sont incapables de vivre en leur absence, ou facultatifs dans le cas contraire. L'étude de ces symbiotes passe souvent par l'utilisation d'antibiotiques, dont les effets sur les communautés microbiennes

d'insecte ne sont pas bien connus, et il a été montré que les symbiotes des insectes peuvent contribuer à une meilleure fécondité, longévité, résistance de leur hôte contre des stress abiotiques ou biotiques. Ces symbiotes peuvent vivre aussi bien à la surface de leur hôte sur la cuticule qu'à l'intérieur même des cellules. La transmission des symbiotes chez les insectes peut être verticale par les parents, ou horizontale par contamination avec des congénères ou par l'environnement (ex. le sol ou la plante hôte).

Les microorganismes peuvent jouer différents rôles au sein des interactions plante-insecte. D'un part, certains microorganismes, qualifiés d'entomopathogènes, sont directement toxiques pour les insectes, et d'autres vont amplifier les défenses des plantes, qui vont ensuite affecter négativement le développement de l'insecte. Ceci a souvent été étudié dans des études n'utilisant qu'une seule souche ou espèce de microorganismes, et des études à l'échelle des communautés entières en font également la preuve. D'autre part, les insectes ont pu évoluer afin de faire face aux défenses des plantes. Il s'avère que certains symbiotes chez les insectes ont la capacité de manipuler, perturber ou supprimer les défenses des plantes, permettant ainsi à leur hôte de se développer.

Dans un contexte agricole, les insectes causent d'importants dommages aux plantes, induisant dans les cas de fortes infestations des pertes conséquentes de rendement et donc financières pour les agriculteurs. Dans un contexte politique de réduction de l'utilisation des pesticides, jusqu'alors principal moyen de lutte, en raison de leur impact sur l'environnement et la santé humaine, la compréhension des interactions biologiques pour contrôler les insectes phytophages semble prometteuse comme nouveau levier de lutte contre les bio-agresseurs, et en particulier l'utilisation de molécules biochimiques ou la manipulation d'organismes biologiques. Ainsi les interactions plante-insecte sont particulièrement étudiées pour mieux comprendre les mécanismes de défenses de la plante contre les insectes et comment ces mécanismes peuvent être amplifiés par la présence ou manipulation de communautés microbiennes.

3. Modèles biologiques

Le colza (*Brassica napus*), de la famille des Brassicacées, est une plante cultivée sur tous les continents qui présente un fort intérêt agronomique. Le microbiote actif des racines et de la rhizosphère de colza sains âgés de 4 semaines, ont été récemment décrits tandis que plusieurs bactéries et champignons habitant le compartiment racinaire ont été identifiés pour leurs activités bénéfiques pour la croissance de leur plante hôte *B. napus* ou contre des

pathogènes. En revanche, peu d'études ont porté sur leurs effets bénéfiques contre des insectes phytophages.

La mouche du chou (*Delia radicum*) est présente dans les milieux tempérés et est un insecte phytophage spécialiste des plantes de la famille des Brassicacées. Alors que son stade adulte est aérien, son stade larvaire cause d'importants dégâts au niveau du compartiment racinaire des plantes. Son microbiote a récemment été décrit au stade larvaire et au stade adulte, mais rien n'est connu du mode de transmission de son microbiote.

4. Principaux objectifs de la thèse et stratégies expérimentales

Cette thèse a pour objectif d'étudier les interactions entre une plante cultivée, un insecte phytophage du compartiment racinaire et leurs microbiotes associés. Elle s'organise en trois parties : i) dans la première partie, nous avons manipulé la diversité microbienne du sol afin d'étudier ses effets sur le développement et l'oviposition de *D. radicum* via une modulation de la chimie de la plante *B. napus* ; ii) dans la deuxième partie, nous avons décrit l'effet de la phytophagie racinaire par *D. radicum* sur la chimie des racines et les communautés microbiennes des racines et de la rhizosphère de *B. napus* ; iii) dans la troisième partie, nous avons analysé les communautés bactériennes de *D. radicum* au stade adulte en présence d'antibiotiques et durant son développement pour déterminer le mode de transmission (verticale ou horizontale) de son microbiote.

Chapitre II – Communautés microbiennes du sol : modulateurs de la chimie des plantes et des traits d'histoire de vie de l'insecte ?

Papier 1 – La diversité microbienne du sol peut-elle influencer les métabolites de la plante et les traits d'histoire de vie d'un insecte rhizophage? Une démonstration chez le colza.

Les interactions entre les plantes et les insectes phytophages jouent un rôle important dans la composition biochimique des plantes. Réciproquement, les métabolites végétaux peuvent influencer les traits d'histoire de vie de ces insectes et contribuer largement à leur valeur sélective (i.e. survie et fécondité). Les microorganismes de la rhizosphère représentent un facteur biotique important qui module la production de métabolites et l'adaptation aux stress des plantes. Les interactions plante-insectes ou plante-microorganismes et leurs conséquences sur la signature métabolomique de la plante sont documentées, mais l'influence des

communautés microbiennes du sol sur les défenses des plantes contre les insectes phytophages reste mal connu.

Dans une première étude, nous avons étudié l'influence des communautés microbiennes du sol sur les défenses de *B. napus* contre un insecte phytophage *D. radicum*. Nous avons testé si la diversité microbienne du sol influençait le taux d'émergence, la taille et l'oviposition de l'insecte et nous avons essayé de relier ces potentiels effets à des modifications des métabolites primaires et secondaires des feuilles et des racines. Pour cela, nous avons manipulé la diversité microbienne du sol en utilisant une approche de dilution jusqu'à extinction de sorte à obtenir un gradient de diversités à inoculer dans une même matrice de sol stérile, dans lesquels les plantes se sont développées. Trois modalités microbiennes de sol (« forte », « medium » et « faible ») ont été obtenues et évaluées grâce à un séquençage par Illumina MiSeq en utilisant les amorces 16S et 18S pour l'analyse des communautés bactériennes et fongiques respectivement.

Notre approche expérimentale nous a permis d'obtenir un gradient décroissant de diversité microbienne du sol. La modalité « medium » dans la rhizosphère a significativement amélioré le taux d'émergence tandis que la taille et l'oviposition n'ont pas été influencées. En revanche, les différentes modalités microbiennes de sol n'ont pas modulé les profils métabolomiques des feuilles et des racines qui auraient pu expliquer les résultats obtenus sur la mouche. Mais la condition d'infestation par *D. radicum* a diminué les teneurs totales en acides aminés et glucosinolates dans les feuilles et dans les racines, ainsi que la teneur totale en sucre dans les racines, comparée à la condition témoin. Nous avons donc mis en évidence les effets potentiels des communautés microbiennes du sol sur les traits d'histoire de vie de *D. radicum*, mais nous n'avons pas été en mesure d'identifier les mécanismes sous-jacents responsables de ces changements.

Short note 1 – La diversité microbienne du sol peut-elle influencer le comportement adulte d'un insecte rhizophage par le biais d'une modification des indices visuels et olfactifs des plantes?

Dans une seconde étude, nous avons étudié les effets de la diversité microbienne du sol sur le comportement de *D. radicum* et plus précisément sur sa capacité à choisir un site de ponte, et si son choix pouvait être expliqué par des signaux visuels ou olfactifs émis par *B. napus*, mesurés à différents stades de développement de la plante. Comme précédemment, une approche de dilution jusqu'à extinction a permis d'obtenir les trois modalités microbiennes de sol (« forte », « medium » et « faible ») dans lesquelles les plantes se sont développées durant

4 et 6 semaines. L'oviposition de *D. radicum* a été monitorée dans une expérience de choix. La teneur en chlorophylle (signal de couleur verte) et la biomasse des plantes ont été mesurées, et la composition en COVs foliaires analysée.

Nous avons observé que la diversité microbienne du sol influençait l'oviposition de *D. radicum* et que la préférence des femelles changeait avec le développement de la plante : la modalité « faible » lorsque les plantes étaient âgées de 4 semaines et la modalité « medium » lorsqu'elles étaient âgées de 6 semaines. Ces résultats n'ont pas pu être reliés au phénotype de la plante puisque les biomasses foliaires et racinaires ainsi que la composition des COVs n'étaient pas affectées par la diversité microbienne du sol, et la teneur en chlorophylle que très faiblement.

Afin de développer notre compréhension du comportement de *D. radicum*, différents signaux de la plante devront être évalués, tels que les glucosinolates, des composés de surface connus pour stimuler l'oviposition. Enfin, dissocier les composés chimiques associés aux plantes de ceux associés aux microorganismes pourraient nous aider à appréhender les effets de chaque acteur dans l'étude d'interaction tripartite.

Chapitre III – Phytophagie racinaire : facteur de la chimie des plantes et des communautés microbiennes racinaires ?

Papier 2 – Influence de la phytophagie racinaire sur la dynamique des communautés microbiennes racinaires et de la rhizosphère

Des études récentes se sont focalisées sur les effets des microorganismes des racines et de la rhizosphère sur les interactions plante-insecte phytophage et ont graduellement changé notre perception de la capacité des microorganismes à affecter les défenses des plantes, mais l'effet inverse a rarement été examiné. Notre étude a eu pour but de déterminer comment la phytophagie influence la dynamique des communautés microbiennes des racines et de la rhizosphère, et si des changements potentiels dans les compositions en métabolites et éléments chimiques des racines, produits durant la phytophagie, pouvaient être reliés à la diversité microbienne des communautés.

Différentes diversités microbiennes ont été obtenues avec des approches de dilution jusqu'à extinction pour être inoculées dans une même matrice de sol stérile. L'échantillonnage des racines et de la rhizosphère a été réalisé à différents stades clés du développement de *D. radicum* correspondant à des intensités de perturbation différentes : éclosion, troisième stade

larvaire, fin de l'émergence. Les échantillons des racines et de la rhizosphère ont été ensuite séquencés par Illumina MiSeq en utilisant les amorces 16S et 18S pour l'analyse des communautés bactériennes et fongiques respectivement. Les teneurs en métabolites primaires et secondaires ainsi qu'en éléments chimiques ont été mesurées sur les échantillons racinaires.

Les communautés bactériennes racinaires ont été plus affectées par la phytophagie que certains phyla bactériens de la rhizosphère ou que les communautés fongiques, qui semblaient plus résistantes à cette perturbation. La phytophagie racinaire a augmenté l'abondance relative du phylum des γ -Proteobactéries dans les racines et la rhizosphère, ainsi que le phylum des Firmicutes dans la rhizosphère. La phytophagie avait tendance à diminuer la teneur de la plupart des acides aminés et sucres racinaires, mais à augmenter les teneurs en tréhalose, en glucosinolates indoles et en soufre. Des abondances plus élevées de quatre genres bactériens (*Bacillus*, *Paenibacillus*, *Pseudomonas* et *Stenotrophomonas*) étaient associées durant la phytophagie à l'augmentation du tréhalose et de composés soufrés. Nous avons proposé le scénario selon lequel en présence de phytophagie, les plantes produiraient des composés défensifs mais recruteraient aussi avec des composés chimiques attractifs, des microorganismes qui permettraient à la plante de maintenir ses défenses.

Des recherches plus approfondies sont nécessaires pour identifier les fonctions biologiques des genres microbiens influencés par la phytophagie et leurs potentielles implications dans les défenses des plantes.

Chapitre IV – Communautés bactériennes d'un insecte phytophage du compartiment racinaire

Papier 3 – Effets à long terme des antibiotiques sur les communautés bactériennes de mouches adultes

Les symbiotes bactériens d'insectes profitent grandement à leur hôte (i.e. amélioration de leur valeur sélective ou de leur résistance à des stress biotiques ou abiotiques). L'étude des symbiotes chez les insectes repose sur l'utilisation d'antibiotiques à large spectre comme la tétracycline pour affaiblir ou supprimer les communautés symbiotiques. Alors que les antibiotiques sont connus pour avoir une influence négative sur le développement des insectes, leur effet précis sur les communautés microbiennes d'insectes et la transmission de leur effet sur plusieurs générations n'est pas renseigné. Nous avons caractérisé les communautés bactériennes de *D. radicum* au stade adulte dans une population exempte de *Wolbachia* (un

symbionte facultatif), évalué l'effet direct de la tétracycline sur ces communautés et analysé si des effets étaient encore visibles après deux générations de traitements antibiotiques successifs.

Notre expérience a impliqué trois générations de *D. radicum* où des mouches des première et deuxième générations ont ingéré de la tétracycline ou non, tandis que les mouches de la troisième génération n'étaient pas traitées mais se distinguaient par le fait que leurs parents et leurs grands-parents avaient été traités ou non. Les communautés bactériennes de mouches adultes ont été analysées à l'aide d'amorces 16S et séquencées par Illumina MiSeq.

Nous avons montré que la tétracycline diminuait la diversité bactérienne chez *D. radicum* de 37%, mais pas au point d'obtenir des insectes stérilisés (c'est-à-dire aposymbiotiques). Le traitement a induit également des modifications de l'abondance et de la fréquence relative des bactéries. Des effets liés à l'antibiotique étaient encore visibles, tels que des différences de structure des communautés ou de composition, chez les descendants non traités dont les parents et/ou les grands-parents avaient été traités. Les mouches ayant des antécédents d'antibiotiques partageaient des genres bactériens (i.e. *Comamonas*, *Acinetobacter*, *Pseudomonas*), potentiellement résistants à la tétracycline et transmissibles.

Pour aller plus loin, il convient d'étudier la transmission bactérienne chez *D. radicum* afin de déterminer si elle se fait de manière verticale, horizontale ou bien les deux, en comparant respectivement plusieurs stades de développement de l'insecte et l'insecte aux différents compartiments de sa plante hôte avec lequel il interagit.

Papier 4 – Héritabilité et acquisition de bactéries chez un insecte rhizophage

Les insectes hébergent des microorganismes, appelés symbiotes, car certains d'entre eux peuvent procurer des avantages à leurs hôtes. La transmission des symbiotes est variable car ils peuvent être acquis à partir des parents (c.-à-d. par transmission verticale), de l'environnement (c.-à-d. par transmission horizontale) ou des deux. Plusieurs études ont démontré la capacité des symbiotes d'insectes à manipuler, perturber ou supprimer la réponse de la plante envers les insectes phytophages. De même, il a été découvert que *D. radicum* héberge des bactéries dans l'intestin des larves, qui portent un gène responsable de la dégradation de l'isothiocyanate, une molécule de défense produite par les plantes. Notre étude visait à évaluer la transmission verticale et horizontale des bactéries chez *D. radicum* en utilisant une approche de métabarcodage à haute résolution basée sur des amorces *gyrB* et un séquençage par Illumina MiSeq.

Pour ce faire, nous avons comparé plusieurs stades de développement de *D. radicum* afin d'évaluer la transmission verticale et les stades larvaires aux compartiments racinaires et de la rhizosphère de *B. napus* afin d'évaluer la transmission horizontale.

Notre étude a montré que la structure et la composition des communautés bactériennes variaient selon les stades de développement de *D. radicum*. Nous avons aussi observé plusieurs espèces bactériennes (*Serratia marcescens* et *Acinetobacter guillouiae*) qui étaient partagées par les femelles et les œufs, suggérant une possible transmission verticale, ainsi que d'autres espèces (*Pseudomonas* et *Lacibacter cauensis*) présentes à la fois dans les larves et dans les racines et la rhizosphère, reflétant une potentielle transmission horizontale. De manière intéressante, les genres *Serratia*, *Acinetobacter* et *Pseudomonas* ont également été identifiés dans la littérature comme porteurs d'un gène *saxA* responsable de la détoxification des défenses de la plante.

Pour aller plus loin, des approches fonctionnelles sont nécessaires, en utilisant des outils pour localiser ces acteurs identifiés comme la microscopie et prédire les fonctions du microbiote comme par exemple des approches métatranscriptomiques, afin de mieux comprendre le rôle des bactéries transmises verticalement ou horizontalement à leur insecte hôte et d'optimiser ainsi la lutte biologique à l'aide d'agents microbiens.

Chapitre V – Discussion générale et perspectives

L'originalité de cette thèse vient du fait que nous nous sommes intéressés à l'interaction entre une plante cultivée (*B. napus*) et un insecte phytophage du compartiment racinaire (*D. radicum*), tout en prenant en compte leurs communautés microbiennes associées, créant ainsi une interaction quadripartite ou une interaction entre deux holobiontes. Nous avons montré que les microorganismes du sol peuvent effectivement servir de levier pour influencer le développement et le comportement d'un insecte phytophage du compartiment racinaire, dont il a été démontré que la phytophagie avait une influence très importante sur la chimie des plantes et sur les communautés microbiennes des racines et de la rhizosphère. Bien que l'utilisation d'antibiotiques ait des effets durables sur les communautés microbiennes et le phénotype d'insectes, viables malgré le traitement, cela laisse penser que les bactéries restantes se transmettent avec succès au fil des générations, contribuant potentiellement à la survie des insectes. Les transferts verticaux et horizontaux de microorganismes contribuent à façonner les

communautés microbiennes de *D. radicum* et nous spéculons sur le rôle joué par les bactéries transmises dans la détoxification des défenses des plantes.

Dans notre première partie, nous avons montré que les microorganismes du sol peuvent influencer les traits d'histoire de vie d'un insecte phytophage, mais les mécanismes sous-jacents sont encore à élucider, en identifiant par exemple les composés chimiques de la plante affectés par ces microorganismes. L'évolution des outils chimiques permet dorénavant l'identification et la quantification d'un certain nombre de métabolites secondaires (c.-à-d. glucosinolates), qui étaient auparavant difficilement mesurables. De plus, le sol est un facteur très important dans la structuration des communautés microbienne du sol mais aussi des racines, d'où l'importance de confirmer nos résultats en utilisant des communautés microbiennes de sols différents. Dans notre deuxième partie, nous avons montré que la phytophagie modifiait les communautés microbiennes des racines et de la rhizosphère, potentiellement au travers de modifications chimiques des racines. Sans pouvoir le démontrer dans notre étude, il est en revanche admis dans la littérature que l'émission d'exsudats racinaires ou de COVs par les racines contribuent à structurer les communautés microbiennes, dont les interactions varient en fonction des stress subis par la plante comme la phytophagie. Dans notre troisième partie, l'application d'un antibiotique a modifié les communautés bactériennes de la génération ayant subi ce traitement ainsi que des générations ultérieures. Les effets de l'antibiotique ont également été observés sur les traits d'histoire de vie de la dernière génération qui n'avait pas été traitée, ce qui soulève la question de la transmission bactérienne chez *D. radicum*. Bien que nous ayons observé plusieurs espèces bactériennes partagées entre la femelle et les œufs et entre les larves et les racines, connues dans la littérature pour avoir un rôle dans la détoxification des défenses des plantes, seul le marquage et l'étude de l'expression des gènes bactériens permettraient de confirmer les voies de transmission des bactéries et d'identifier leurs fonctions.

Avoir une meilleure compréhension des interactions plantes-insectes et de la force avec laquelle les microorganismes peuvent influencer leur propre hôte ou d'autres organismes en interaction est une étape cruciale qui pourrait promouvoir les applications microbiennes et améliorer leur efficacité dans la lutte biologique contre les insectes.



Endosymbiosis: Homage to Lynn Margulis,
a painting by Shoshanah Dubiner (Guerrero *et al.*, 2013)

Titre : Contribution à l'analyse des interactions tripartites entre *Brassica napus*, *Delia radicum* et leur microbiote

Mots clés : colza, mouche du chou, communautés microbiennes, métabolites, tétracycline, transmissions verticale et horizontale

Résumé : Les microorganismes exercent une forte influence sur les interactions plante-insecte. Nous avons étudié l'interaction entre le colza (*Brassica napus*), la mouche du chou (*Delia radicum*) et les communautés microbiennes qui leur sont associées. La diversité microbienne du sol a été manipulée par dilution jusqu'à extinction pour évaluer son effet sur la chimie de la plante et les traits d'histoire de vie de l'insecte. La différence de diversité a influencé le taux d'émergence et l'oviposition de la mouche, mais pas la chimie de la plante. A l'inverse, la phytophagie par *D. radicum* a drastiquement modifié la chimie des racines et les communautés microbiennes des racines et de la rhizosphère. Nous avons proposé le scénario selon lequel les plantes soumises à des attaques de phytophages produiraient des composés défensifs mais recruteraient aussi avec des composés chimiques attractifs, des microorganismes du sol qui permettraient à la plante de maintenir ses

défenses. Les communautés bactériennes des mouches adultes, sans leur symbiote facultatif *Wolbachia*, ont été étudiées à l'aide d'un antibiotique. Nous avons montré que la tétracycline diminuait la diversité bactérienne des mouches sans les rendre stériles, modifiait la composition des communautés, et que les effets étaient durables sur plusieurs générations. Enfin, l'étude de la transmission des bactéries chez *D. radicum* a montré deux espèces partagées entre les femelles et les oeufs, et deux autres entre les larves et les racines et le sol. Cette étude montre qu'avoir une meilleure compréhension des interactions plantes-insectes et de la grande influence des microorganismes sur leur propre hôte ou d'autres organismes en interaction est une étape cruciale qui pourrait promouvoir les applications microbiennes dans un contexte de lutte biologique contre les insectes.

Title: Contribution to the analysis of tripartite interactions between *Brassica napus*, *Delia radicum* and their microbiota

Keywords: oilseed rape, cabbage root fly, microbial communities, metabolites, tetracycline, vertical and horizontal transmission

Abstract: Microorganisms have a strong influence on plant-insect interactions. We have studied the interaction between oilseed rape (*Brassica napus*), the cabbage root fly (*Delia radicum*) and their associated microbial communities. Soil microbial diversity was manipulated with the dilution to extinction approach to assess its effect on plant chemistry and insect life history traits. Diversity variation influenced the fly emergence rate and oviposition, but not plant chemistry. Conversely, herbivory by *D. radicum* strongly modified root chemistry and both root and rhizosphere microbial communities. We proposed a scenario that in the presence of herbivory, plants would produce defensive compounds but also would recruit, with attractive chemical compounds, soil microorganisms

that may maintain plant defenses. Bacterial communities of adult flies, free of their facultative symbiont *Wolbachia*, were studied using an antibiotic. We showed that tetracycline decreased fly bacterial diversity, without making them sterile, modified community composition, and that effects lasted over several generations. Lastly, studying bacterial transmission in *D. radicum* showed two species shared between females and eggs, and two others between larvae and both roots and rhizosphere. This study showed that having a better understanding of plant-insect interactions and how strongly microorganisms can influence their own host or other interacting organisms is a crucial step that could promote microbial applications in a context of insect biological control.