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Research Article

RESEARCH ARTICLE

Host origin and tissue microhabitat shaping the microbiota of the terrestrial isopod *Armadillidium vulgare*

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One sentence summary: This is the first in-depth study of the bacterial communities associated with different tissues of a terrestrial isopod crustacean, showing that microbiota composition is strongly influenced by host origin.
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ABSTRACT

We present the first in-depth investigation of the host-associated microbiota of the terrestrial isopod crustacean *Armadillidium vulgare*. This species is an important decomposer of organic matter in terrestrial ecosystems and a major model organism for arthropod-*Wolbachia* symbioses due to its well-characterized association with feminizing *Wolbachia*. 16S rRNA gene pyrotags were used to characterize its bacterial microbiota at multiple levels: (i) in individuals from laboratory lineages and field populations and (ii) in various host tissues. This integrative approach allowed us to reveal an unexpectedly high bacterial diversity, placing this species in the same league as termites in terms of symbiotic diversity. Interestingly, both animal groups belong to the same ecological guild in terrestrial ecosystems. While *Wolbachia* represented the predominant taxon in infected individuals, it was not the only major player. Together, the most abundant taxa represented a large scope of symbiotic interactions, including bacterial pathogens, a reproductive parasite (*Wolbachia*) and potential nutritional symbionts. Furthermore, we demonstrate that individuals from different populations harboured distinct bacterial communities, indicating a strong link between the host-associated microbiota and environmental bacteria, possibly due to terrestrial isopod nutritional ecology. Overall, this work highlights the need for more studies of host–microbiota interactions and bacterial diversity in non-insect arthropods.

Keywords: bacterial diversity; *Hepatoplasma*; host–symbiont interactions; microbiota; symbiosis; *Wolbachia*

INTRODUCTION

In recent years, heritable bacterial symbionts have been recognized as essential players in arthropod ecology and evolution. For instance, numerous insects harbour consortia of highly specialized symbiotic bacteria, consisting of coevolved obligate mutualistic endosymbionts (primary symbionts) as well as facultative (or secondary) symbionts (Moran, McCutcheon and Nakabachi 2008; Moya *et al.* 2008; Koga *et al.* 2013). Together, these bacterial symbionts have synergistic effects on host fitness, ranging from the provisioning of essential nutrients lacking from nutrient-deficient diets such as plant sap (e.g. aphids

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or whiteflies) or vertebrate blood (e.g. tsetse flies) (Thao and Baumann 2004; Moran, Dunbar and Wilcox 2005; Pais *et al.* 2008) to increased thermal tolerance, host plant speciation, predator avoidance and defence against natural enemies and parasites (Montllor, Maxmen and Purcell 2002; Oliver *et al.* 2003; Ferrari *et al.* 2004; Tsuchida, Koga and Fukatsu 2004; Oliver, Moran and Hunter 2005; Scarborough, Ferrari and Godfray 2005; Jaenike *et al.* 2010; Tsuchida *et al.* 2010; Lukasik *et al.* 2013).

In contrast, other facultative bacteria establish stable symbiotic associations without conferring an obvious benefit to their host. To avoid elimination from host populations, these bacteria use a different strategy and act as reproductive parasites (Duron *et al.* 2008). Being maternally transmitted, they have developed various mechanisms (i.e. cytoplasmic incompatibility, parthenogenesis, male killing and feminization of genetic males) to manipulate host reproduction to promote their own vertical transmission (Stouthamer *et al.* 1993; Hurst, Jiggins and Majerus 2003; Bouchon, Cordaux and Grève 2008; Serbus *et al.* 2008). Within this group, bacteria of the genus *Wolbachia* remain the best studied and the most frequently encountered reproductive manipulators with the largest spectrum of phenotypes (Hilgenboecker *et al.* 2008; Werren, Baldo and Clark 2008; Sicard *et al.* 2014).

This diversity of symbiont-mediated effects on hosts has led to a recent shift towards a more holistic, community-based approach in symbiosis research, including the perception of a host together with its associated microbiota as a functional entity, also referred to as the 'holobiont' (Zilber-Rosenberg and Rosenberg 2008; Gilbert *et al.* 2010; Feldhaar 2011; Brucker and Bordenstein, 2012, 2013). From this perspective, the association with a stable, heritable symbiotic bacterial community not only complements the host's metabolic capabilities but also represents a source of evolutionary novelty. This highlights the need to consider a host as well as its associated microbiota to fully understand the multipartite host symbiont–microbiota interactions that shape complex symbiotic associations.

While a large body of work on arthropod-associated microbiotas has been conducted in insects, terrestrial isopod crustaceans represent an excellent model system for understanding of complex multipartite symbioses. First, they feed on dead and decaying organic matter and therefore represent keystone species as plant litter decomposers in many terrestrial ecosystems (Zimmer *et al.* 2002b, 2004; Hornung 2011). This places them in the same ecological guild as the extensively studied termites, which rely on a diverse hindgut community of symbiotic bacteria, archaea and (in the lower termites) protozoans for the efficient degradation of lignocellulose in the ingested plant material (Brune and Stingl 2005; Todaka *et al.* 2007; Warnecke *et al.* 2007; Hongoh 2010; Brune 2014). Along the same lines, it has been hypothesized that bacteria located in the midgut caeca of terrestrial isopods might play a role in digestive processes (e.g. via bacterial cellulolytic enzymes) and that the acquisition of these bacteria might have been a prerequisite for the colonisation of land by isopods (Zimmer *et al.* 2001, 2002a; Zimmer and Bartholmé 2003; Wang, Brune and Zimmer 2007). While several bacteria have indeed been observed in the digestive tissues of numerous terrestrial isopod species, including the caeca-associated taxa *Candidatus* Hepatoplasma crinochetorum and *Candidatus* Hepatincola porcellionum (Wang *et al.* 2004a,b; Kostanjsek, Strus and Avgustin 2007; Wang, Brune and Zimmer 2007), the precise nature of their association with terrestrial isopods remains to be elucidated. Second, the terrestrial isopod *Armadillidium vulgare* represents a major model system in the context of arthropod-*Wolbachia* symbioses due to its well-characterized association with feminizing *Wolbachia*. Three different feminizing *Wolbachia* strains (*w*VulC, *w*VulM and *w*VulP) occur in this species, establishing stable single infections (Rigaud *et al.* 1991; Cordaux *et al.* 2004; Verne *et al.* 2007, 2012). However, the full scope of bacterial symbionts associated with this host has never been investigated on a large scale. As a first step towards that goal, a recent quantitative investigation of both *Wolbachia* and overall bacterial titers across several tissues of *A. vulgare* has revealed that *Wolbachia* is part of a potentially more diverse bacterial community (Dittmer *et al.* 2014).

In this study, we build upon these previous findings and provide a more complete picture of the microbiota associated with *A. vulgare*. To this end, 16S rRNA gene amplicon pyrosequencing was used to characterize the bacterial communities of *A. vulgare* from both laboratory lineages and field populations, including *Wolbachia*-infected and uninfected individuals. Moreover, the symbiotic communities were analysed in five major host tissues, according to the notion that a host organism represents an ecosystem composed of different tissue microhabitats for bacteria and that the latter might therefore contain tissue-specific bacterial communities. Considering that the feeding habits (detritivory and coprophagy) of terrestrial isopods provide ample opportunities to encounter environmental bacteria and/or maintain stable associations with particular taxa (e.g. associated with their food sources or faeces), bacterial communities from faeces and soil samples were also included to investigate potential links between the host-associated microbiota and environmental bacterial consortia. This integrative approach allowed us to (i) reveal an unexpectedly high bacterial diversity, (ii) identify the predominant bacterial symbionts and (iii) determine the major factors shaping microbiota composition in this species.

MATERIALS AND METHODS

Animal and soil samples

Animals used for this work were sampled from four different laboratory lineages and three field populations. They were partly the same as in our previous quantitative study (Dittmer *et al.* 2014) and are summarized in Table 1. In the laboratory, animals were kept at 20°C and natural photoperiod in plastic breeding boxes on moistened potting mix and fed *ad libitum* with lime

Table 1. Origin and infection status of specimens used for amplicon sequencing. M = Males, F = Females.

Origin	Lineage/population	Gender (infection status)	N
Laboratory	*Wolbachia*-free lineage	M	10
		F	10
	C lineage	F (*w*VulC)	10
	M lineage	F (*w*VulM)	10
	P lineage	F (*w*VulP)	10
Field	Availles	M	6
		F (*w*VulC)	6
		F (*w*VulM)	6
		F (*w*VulC + *Rickettsiella*)	1
	Plaine Mothaise	M	3
		M intersexual (*w*VulC)	1
		F (*w*VulC)	5
	Thuré	M (*w*VulC/*w*VulM)	5
		F (*w*VulC)	5

tree leaves and carrot slices. One laboratory lineage was *Wolbachia* free, that is, both males and females from this lineage do not carry *Wolbachia*. In the three other lineages, natural infections with either of the *Wolbachia* strains *w*VulC, *w*VulM or *w*VulP have been stably maintained for at least 7 years (30 years for the oldest lineage). Since the *Wolbachia* strains infecting this host species are feminizers, only females from these lineages harbour *Wolbachia*, while males are expected to be uninfected. A total of 10 males and 10 females (pairs of brothers and sisters) were randomly chosen from the *Wolbachia*-free lineage, as well as 10 females each from the *w*VulC, *w*VulM and *w*VulP lineages. In addition to the animal samples, soil substrate samples were taken in 1.5 ml tubes from four breeding boxes per lineage.

In addition, wild specimens were sampled from three locations in the region Poitou-Charentes (France) in autumn 2011 and 2012: Availles-Thouarsais (46° 51′ 37″ N, 0° 8′ 28″ E) and Thuré (46°49′59″ N, 0°27′37″ E) are located in an agricultural landscape used for cereal crop farming, while the Plaine Mothaise is a wetland area used for cattle grazing (46° 21′ 21″ N, 0° 06′ 32″ E). The collected specimens were kept in plastic boxes with soil and leaves from their respective sampling site until dissection. As for the laboratory lineages, two replicate soil samples were collected simultaneously in 1.5 ml tubes from Availles and Thuré, but not from the Plaine Mothaise.

DNA extraction

Twenty-four hours before dissection, individuals were transferred into separate boxes without potting mix or soil from the field to collect their faeces for DNA extraction. A total of 15 faeces were sampled from each individual and immediately frozen at –20°C. DNA from faeces and soil was extracted using the NucleoSpin Soil Kit (Macherey-Nagel) according to the manufacturer's instructions. Prior to dissection, individuals were surface sterilized using sodium hypochlorite and haemolymph was collected after piercing the cuticle with a sterile needle. The following tissues were then dissected out using sterilized instruments: gonads, nerve cord, midgut caeca and hindgut. All tissues were rinsed in Ringer solution to avoid cross-contamination between tissues. Additionally, the hindgut was cut longitudinally and washed in Ringer solution to retain only the hindgut tissue without its contents. DNA was extracted from all tissues using phenol–chloroform (Kocher *et al.* 1989). DNA yield and quality was assessed using the NanoDrop spectrophotometer and all samples were diluted to approximately 15 ng μl^{-1}.

Wolbachia infection status

Wolbachia infection status and *Wolbachia* titers in all host tissues included in this study have been determined previously for all individuals from laboratory lineages, Availles and the Plaine Mothaise via diagnostic as well as quantitative PCR of the *wsp* gene (see Dittmer *et al.* 2014 for details), and the same qPCR approach was used here for the samples from Thuré (Table S1, Supporting Information). All but two females from Availles were infected with *Wolbachia*, harbouring either *w*VulC (N = 7) or *w*VulM (N = 17). Among the individuals from the Plaine Mothaise, all females were infected with *w*VulC (N = 5). In addition, one male was identified as an intersexual: while the external morphological characters were male, the androgenic glands were hypertrophied, which is an indication of *Wolbachia* infection with incomplete feminization (Legrand and Juchault 1969; Legrand, Juchault and Mocquard 1974). Indeed, diagnostic PCR and subsequent sequencing confirmed that, like *Wolbachia*-infected females from the same population, this male harboured *w*VulC, albeit at lower titers in most host tissues (Table S1, Supporting Information). Except for this intersexual male, *Wolbachia* infection patterns in laboratory lineages and these two field populations were therefore typical for feminizing *Wolbachia*, that is, males are uninfected while females exhibit high *Wolbachia* titers in all tissues (Dittmer *et al.* 2014).

In contrast, the *Wolbachia* infection status of the individuals from Thuré was clearly different: both males (N = 9) and females (N = 9) were positive for *Wolbachia* based on quantitative PCR, while the infection was undetectable in most individuals using standard PCR procedures. However, only two individuals harboured *Wolbachia* in all tissues, while the infection seemed to be restricted to haemolymph and nerve cord in most specimens. Moreover, *Wolbachia* titers were extremely low, the highest ones measured in the haemolymph and nerve cord corresponding to only 4.76% and 1.89%, respectively, of the *Wolbachia* titers previously observed in females stably infected with the *w*VulC strain (Dittmer *et al.* 2014; Table S1, Supporting Information). PCR products that had signals strong enough to be sequenced corresponded to *w*VulC, with the exception of one male that carried *w*VulM.

Detection of *Rickettsiella* infection

One of the *w*VulC-infected females from Availles showed external signs of infection with the bacterial pathogen *Rickettsiella*, that is, a white substance in the body cavity (Cordaux *et al.* 2007; Bouchon, Cordaux and Grève 2011). Three protein-encoding marker genes (*gidA*, *rpsA* and *sucB*) were amplified from the different tissues of this female using *Rickettsiella*-specific primers and PCR conditions as proposed in Leclerque and Kleespies (2012). This allowed us to confirm that the observed symptoms were indeed due to infection with this pathogen and the infected individual was treated as a separate condition in subsequent analyses.

454 amplicon pyrosequencing

A 526-bp-fragment spanning the variable regions V1–V3 of the bacterial 16S rRNA gene was amplified using the universal primers 27F and 520R. Primers were adapted for 454 amplicon library preparation by adding the 454 Adapter A and a 10-bp Multiplex Identifier sequence (MID) to the reverse primer 520R as well as the 454 Adapter B to the forward primer 27F. 25 μl PCR reactions contained 1X Phusion High Fidelity DNA Polymerase Master Mix (New England Biolabs), 0.5 μM of each HPLC-purified primer, 0.75 μl DMSO and 1.5 μl (20-25 ng) of template DNA. PCR cycles were as follows: 98°C for 30 s, followed by 30 cycles of 98°C for 30 s, 50°C for 30 s, 72°C for 30 s and a final extension of 72°C for 10 min.

A total of 94 amplicon pools containing up to 10 biological replicates were sequenced for an in-depth screening of the bacterial communities associated with *A. vulgare* (see Table S2, Supporting Information, for details). These pools corresponded to amplicons from the five host tissues as well as faeces from different host categories (i.e. males, *Wolbachia*-free females and females infected with either of the three *Wolbachia* strains *w*VulC, *w*VulM and *w*VulP) from both controlled laboratory conditions and the three field sites. In addition, amplicons from the tissues and faeces of the intersexual male and the female showing symptoms of *Rickettsiella* infection were sequenced as

separate conditions. Finally, we included 10 amplicon pools from soil samples collected from breeding boxes in the laboratory and two field sites to compare the host-associated microbiota with that in the surrounding environment. Concerning *w*VulM-infected females from Availles, 6 out of the 17 sampled individuals were randomly chosen to equilibrate the number of replicates per amplicon pool between conditions from different field populations (Table S2, Supporting Information). Each amplicon pool was subjected to two rounds of purification using AMPure Beads (Agencourt Bioscience Corporation) and subsequently quantified using PicoGreen (Invitrogen). Several amplicon pools were then combined at equimolar concentrations, resulting in four multiplexed amplicon libraries for emulsion PCR and 454 sequencing. Two libraries were sequenced in two separate runs on a single PicoTiterPlate region on a 454 GS FLX sequencer (Roche, 454 Life Sciences) by GenoScreen (Lille, France). The two other libraries were sequenced in two separate runs on a GS Junior sequencer (Roche, 454 Life Sciences) in the laboratory Ecology and Biology of Interactions (Poitiers, France).

Data analysis of 454 sequences

Sequences were analysed using the QIIME pipeline version 1.7.0 (Quantitative Insights Into Microbial Ecology, http://qiime.org/; Caporaso *et al.* 2010b). Briefly, the flowgrams of the four runs were demultiplexed, trimmed and denoised separately with AmpliconNoise (Quince *et al.* 2009, 2011), and chimeras were removed using Perseus (Quince *et al.* 2011). The output files were merged, all sequences shorter than 250 bp were discarded and the remaining reads were clustered into operational taxonomic units (OTUs) with 97% similarity using uclust (Edgar 2010). Representative sequences from each OTU were aligned against the Silva reference alignment (release 108; Quast *et al.* 2013) using PyNAST (Caporaso *et al.* 2010a) and identified using the RDP Classifier (Wang *et al.* 2007). Rare OTUs represented by <10 sequences were not included in subsequent analyses. Considering that the different sample types (e.g. host tissues vs soil) are very different in terms of OTU richness and diversity, this cut-off was chosen as a compromise to eliminate OTUs that might represent sequencing artefacts, while keeping rare but potentially biologically meaningful taxa. More stringent cut-offs were tested but produced the same results (data available upon request). Rarefaction curves of the number of observed OTUs were generated after subsampling of 1000 sequences per sample. OTU richness and diversity in each sample were determined using the non-parametric species richness estimator Chao1 and the Shannon Index of diversity, again after subsampling of 1000 sequences per sample. Alpha diversity indices were compared between conditions using two-sample *t*-tests. Bacterial community composition was investigated using principal coordinates analysis (PCoA) based on both unweighted and weighted UniFrac distances (Lozupone and Knight 2005) and network-based analyses using the Edge-weighted spring-embedded algorithm in Cytoscape (version 2.8.3) to illustrate OTU partitioning depending on host populations and tissues. In addition, relationships between bacterial communities at the host individual level were examined using a neighbour-joining tree based on unweighted UniFrac distances between bacterial communities. The OTU corresponding to *Wolbachia* was removed from the dataset prior to the latter analysis as it represented a confounding factor when investigating the impact of *Wolbachia* infection on bacterial community composition.

Microbial co-occurrence networks

Microbial networks representing non-random co-occurrence patterns between OTUs were constructed to further investigate bacterial community structure within host-associated microbiotas from different host populations. To achieve this, bacterial abundance matrices were created separately depending on host origin (i.e. laboratory lineages and each of the field sites) and OTUs containing <10 sequences were removed from each matrix to reduce network complexity. All possible Spearman's rank correlations were then calculated between the remaining OTUs in each matrix. Co-occurrences between bacterial taxa were considered robust when the Spearman's correlation coefficient was both >0.6 and statistically significant ($P < 0.01$) after Bonferroni correction (Barberan *et al.* 2012; Boutin *et al.* 2013). Networks illustrating all valid co-occurrences depending on host origin were built in R (R Project 2.15.1) using the Igraph package. Within these networks, nodes represent different OTUs and connections (edges) correspond to significant correlations between these OTUs.

Predictive functional profiles from reconstructed metagenomes

To complement our taxonomy-based bacterial inventory with additional information regarding the functional gene content present in different bacterial communities, we reconstructed predictive metagenomes from our 16S rRNA gene dataset using PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States, http://picrust.github.io/picrust/; Langille *et al.* 2013). This computational tool infers functional gene content for all bacterial taxa based on the genes present in their closest relative for which the genome has been sequenced. The outcome is a reliable prediction of functional metagenome composition in the absence of actual metagenomic shotgun sequencing data. The accuracy of the predicted metagenomes was verified by calculating the NSTI (Nearest Sequenced Taxon Index) for all samples (Langille *et al.* 2013). The predicted genes were then assigned to KEGG pathways and the reconstructed functional profiles were statistically compared depending on host origin or host tissue using Statistical Analysis of Metagenomic Profiles (STAMP; Parks *et al.* 2014). We used a two-sided Welch's *t*-test for two-group comparisons and ANOVA combined with Tukey's post hoc multiple comparison test with Bonferroni correction for multiple comparisons.

RESULTS

Bacterial diversity and community composition

A total of 501 804 high-quality reads longer than 250 bp were kept after denoising and chimera removal. This resulted in 1615 OTUs represented by ≥10 sequences. On average, 5660 high-quality reads grouped into 153 OTUs were obtained from tissue samples, 2565 reads/309 OTUs from faeces and 1183 reads/268 OTUs from soil samples (see Table S2, Supporting Information, for details). This reveals an astonishing OTU richness in host tissues, indicating that each individual included in the amplicon pools may harbour up to several hundred bacterial taxa. In support of this, the separately sequenced microbiota of the intersexual male consisted of 276 OTUs across the different tissues. The diseased female infected with *Rickettsiella*, on the other hand, harboured only 50 OTUs with *Rickettsiella* as the dominant taxon. Rarefaction curves of the observed OTU richness in 1000

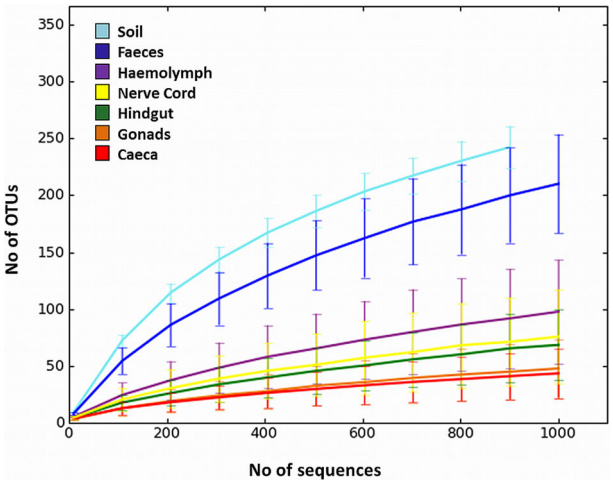

Figure 1. Rarefaction curves of the observed OTU richness in 1000 subsampled sequences from soil, faeces and different host tissues. Error bars represent the SE of the mean number of OTUs obtained from several amplicon pools for each sample type.

Table 2. Richness and diversity indices (mean ± SE) for 1000 subsampled sequences from soil, faeces and different host tissues. Different superscript letters indicate significant differences.

Sample type	Chao 1 Richness estimator	Shannon Diversity index
Soil	359.40 ± 33.74^{a}	7.10 ± 0.26^{a}
Faeces	333.06 ± 67.75^{a}	5.87 ± 0.88^{b}
Haemolymph	184.99 ± 85.63^{b}	3.38 ± 1.31^{c}
Hindgut	146.64 ± 66.64^{b,c}	2.53 ± 1.01^{c}
Nerve cord	141.99 ± 67.75^{b,c}	2.73 ± 1.21^{c}
Gonads	97.95 ± 53.49^{b,c}	1.87 ± 1.17^{c}
Caeca	86.48 ± 46.44^{c}	2.02 ± 0.81^{c}

subsampled sequences showed that our sequencing effort was sufficient to capture the major part of the bacterial diversity present in the various host tissues, while more sequences would probably have added new OTUs to the bacterial communities present in faeces and soil (Fig. 1). OTU richness and diversity, as determined by the species richness estimator Chao 1 and the Shannon Index of diversity, were higher in faeces and soil samples than in host tissues under both laboratory and field conditions (Chao 1: all two-sample *t*-tests $P \leq 0.03$; Shannon: all two-sample *t*-tests $P \leq 0.015$, Table 2). Regarding the different host tissues, OTU richness was higher in the haemolymph than in the caeca (Chao 1: two-sample *t*-test $P = 0.042$) but bacterial diversity was similar across all tissues (Table 2).

The microbiota associated with host tissues, faeces and soil not only differed in terms of bacterial richness and diversity, but also concerning bacterial community composition (Fig. 2). While Proteobacteria represented the most abundant phylum in all three sample types, their proportion was higher in host tissues (ranging from 71% in the caeca to 98% in the gonads) than in faeces (56%) or soil (48%). Most of the Proteobacteria in the different tissues belonged to the Alphaproteobacteria, which, not surprisingly, was mostly due to *Wolbachia* (from 26% in the caeca to 65% in the gonads). Other Alphaproteobacteria in host tissues included *Sphingomonas* and *Candidatus* Hepatincola porcellionum (hereafter referred to as '*Hepatincola*'), the latter representing 8% of the total bacterial communities in the caeca. None of these bacteria was found to be abundant in faeces or soil, where most Alphaproteobacteria remained unclassified or were assigned to a multitude of low-abundance taxa. The host tissue-associated Gammaproteobacteria consisted of *Halomonas*, *Pseudomonas*, *Shewanella* and two different OTUs assigned to the bacterial pathogen *Rickettsiella* (*Rickettsiella grylli* and *Rickettsiella* sp.). *Pseudomonas* also had a relatively important share in faecal bacterial communities (6%), while most of the other Gammaproteobacteria in the faeces and soil could not be further identified.

Apart from Proteobacteria, the class Mollicutes (phylum Tenericutes) was relatively abundant in all host tissues and faeces, particularly so in the caeca, representing 27% of the bacterial community in this tissue (Fig. 2). In all tissues except the hindgut, this class was represented by a single taxon, *Candidatus*

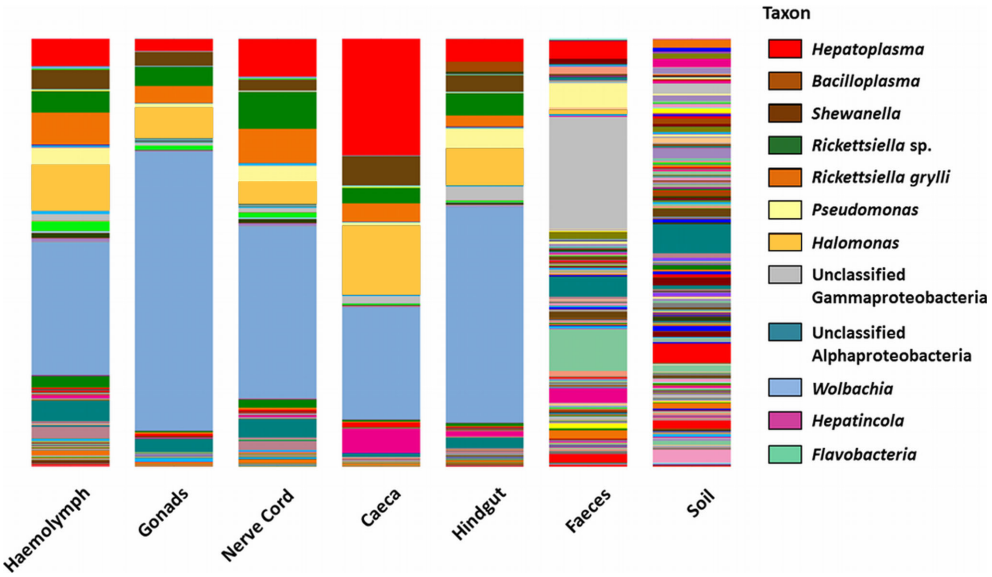

Figure 2. Bacterial community composition (%) in host tissues, faeces and soil. The most abundant bacterial genera are specified in the legend.

Hepatoplasma crinochetorum (hereafter referred to as '*Hepatoplasma*'). In the hindgut, *Hepatoplasma* accounted for 5% of all sequences, while 2% corresponded to *Candidatus* Bacilloplasma. *Hepatoplasma* was also detected in the faeces (4%) but not in the soil. Flavobacteria (phylum Bacteroidetes) were relatively abundant in the faeces (12%) but represented only a minor proportion of the microbiota in different host tissues. Actinobacteria were also more abundant in faeces (18%) and soil (10%) than in host tissues. Other bacterial classes (Acidobacteria, Cytophagia (phylum Bacteroidetes), Sphingobacteria (Bacteroidetes), Gemmatimonadetes and Opitutae (Verrucomicrobia)) occurred at relatively high frequencies in the soil, while being absent or rare in host tissues and faeces.

Predominant members of the host-associated microbiota

Most of the tissue-specific bacterial communities contained a small number of highly represented taxa that dominated the community (Fig. 3). As expected, *Wolbachia* was one of those bacteria, often representing the predominant member of the bacterial communities in various tissues of infected individuals. Hence, in specimens from the laboratory lineages C, M and P as well as Availles and Plaine Mothaise, *Wolbachia* represented at least 60% of the bacterial communities in most tissues (Fig. 3). Not surprisingly, the highest proportions were found in the gonads, where *Wolbachia* represented between 83% and 95% of the bacterial community. A similar pattern was observed for the individually sequenced microbiota of the intersexual male, with *Wolbachia* representing between 43% (haemolymph) and 90% (gonads) of the tissue-specific bacterial communities. The *Wolbachia* infection status of individuals from Thuré was different, with both males and females of this population presenting extremely low *Wolbachia* titers in several (but rarely all) tissues based on qPCR of the *wsp* gene (Table S2, Supporting Information). Nonetheless, *Wolbachia* still represented an important fraction of the microbiota in this population, being present in all tissues of both males and females (Fig. 3). Moreover, a closer inspection of the bacterial community profiles of males from other populations revealed that *Wolbachia* was rarely entirely absent. Hence, *Wolbachia* was detected in the hindgut of males from the laboratory (18% of the bacterial community) as well as in the gonads (45%) and the digestive tissues (17% in the caeca, 36% in the hindgut) of males from Availles (Fig. 3).

Apart from *Wolbachia*, two different OTUs identified in tissue samples from Availles and the Plaine Mothaise were assigned to the bacterial pathogen *Rickettsiella* (Fig. 3). The representative sequences of the two OTUs presented 6.4% sequence divergence over a sequence length of 460 bp and therefore clearly corresponded to two different bacterial strains. One of the OTUs was present in all tissues of the female showing external symptoms of infection and was assigned to *R. grylli*. The second strain was only identified to genus level and is therefore referred to as *Rickettsiella* sp. Additional Blast searches revealed that the former has an identical match in the draft genome of an *R. grylli* strain from an unidentified terrestrial isopod species (Genome Project AAQJ00000000). The second OTU *Rickettsiella* sp. is 98% identical to the 16S rRNA gene of a previously identified *Rickettsiella* symbiont from *A. vulgare* (Accession No. AM490937), for which the pathotype designation '*Rickettsiella armadillidii*' has been proposed (Cordaux *et al.* 2007; Bouchon, Cordaux and Grève 2011). Interestingly, both *Rickettsiella* strains frequently occurred in field samples, while only one individual had presented obvious signs of infection. Sequencing the microbiota of this diseased individual separately revealed that the pathogen was extremely predominant, representing the only taxon present in haemolymph and nerve cord (Fig. 3). In comparison with this individual, *Rickettsiella* accounted for lower proportions of the microbiota in the pooled samples, although still representing up to 60% of the bacterial communities in some tissues of males from the Plaine Mothaise.

The mollicute *Hepatoplasma* and the alphaproteobacterium *Hepatincola* represented two other taxa of particular interest in the bacterial communities analysed here. Despite being facultative, both bacteria have been previously observed in the caeca of various terrestrial isopod species including *A. vulgare* and it has been suggested that they might play a role in digestive processes (Zimmer *et al.* 2001, 2002a; Wang, Brune and Zimmer 2007; Fraune and Zimmer 2008). In our dataset, *Hepatoplasma* was detected in two laboratory lineages (the *Wolbachia*-free and C lineage), while both bacteria were present in the samples from Availles. As expected, *Hepatoplasma* was found to be highly predominant in the caeca (Fig. 3). An unprecedented and interesting finding is that *Hepatoplasma* was not restricted to the caeca, but occurred in all tested host tissues (Fig. 3) as well as in the faeces (Fig. 2), albeit at lower titers. These results have been confirmed via quantitative PCR (Fig. S1, Supporting Information). While *Hepatincola* had a lower share in the microbiotas from Availles than *Hepatoplasma*, it was also observed in all host tissues in *Wolbachia*-infected females but was only found in the caeca in males (Fig. 3).

Another taxon which should be mentioned in the context of bacteria associated with digestive tissues of terrestrial isopods is *Candidatus* Bacilloplasma (Mollicutes), an extracellular bacterium of unknown function which has been previously observed attached to the cuticular spines of the hindgut cuticle of the terrestrial isopod *Porcellio scaber* (Kostanjsek, Strus and Avgustin 2007). Here, *Bacilloplasma* was frequently detected in the hindguts but only represented $\leq$5% of the respective communities, except for males from Thuré (10%). Hence, either *Bacilloplasma* does not occur at high prevalence in *A. vulgare* or most of the bacteria have been eliminated during dissection when removing the hindgut contents.

Link between environmental bacteria and the host-associated microbiota

A high number of OTUs were shared between host-associated bacterial communities (i.e. all tissues confounded) and those from the external environment (faeces and soil), despite the above-mentioned differences in bacterial diversity and community composition between the different sample types. Hence, 51% of all OTUs occurring in host tissues from laboratory-reared specimens also occurred in faeces (13%), soil (8%) or both (30%) (Fig. 4). The situation was similar for samples from field populations, with 59% of all host-associated OTUs also occurring in faeces (30%), soil (2%) or both (27%) (Fig. 4). Nonetheless, PCoA confirmed that host-associated bacterial communities were clearly different from those present in faeces and soil (Fig. 5a). Indeed, the difference between host-associated and external bacterial communities explained 16.23% of the total variability in the dataset. This suggests that the relatively high number of OTUs occurring in all three sample types (tissues, faeces and soil) most likely represent low-abundance OTUs from the external environment, whereas other taxa are more intimately associated with the host environment and contribute to the composition of a distinct, host-associated microbiota.

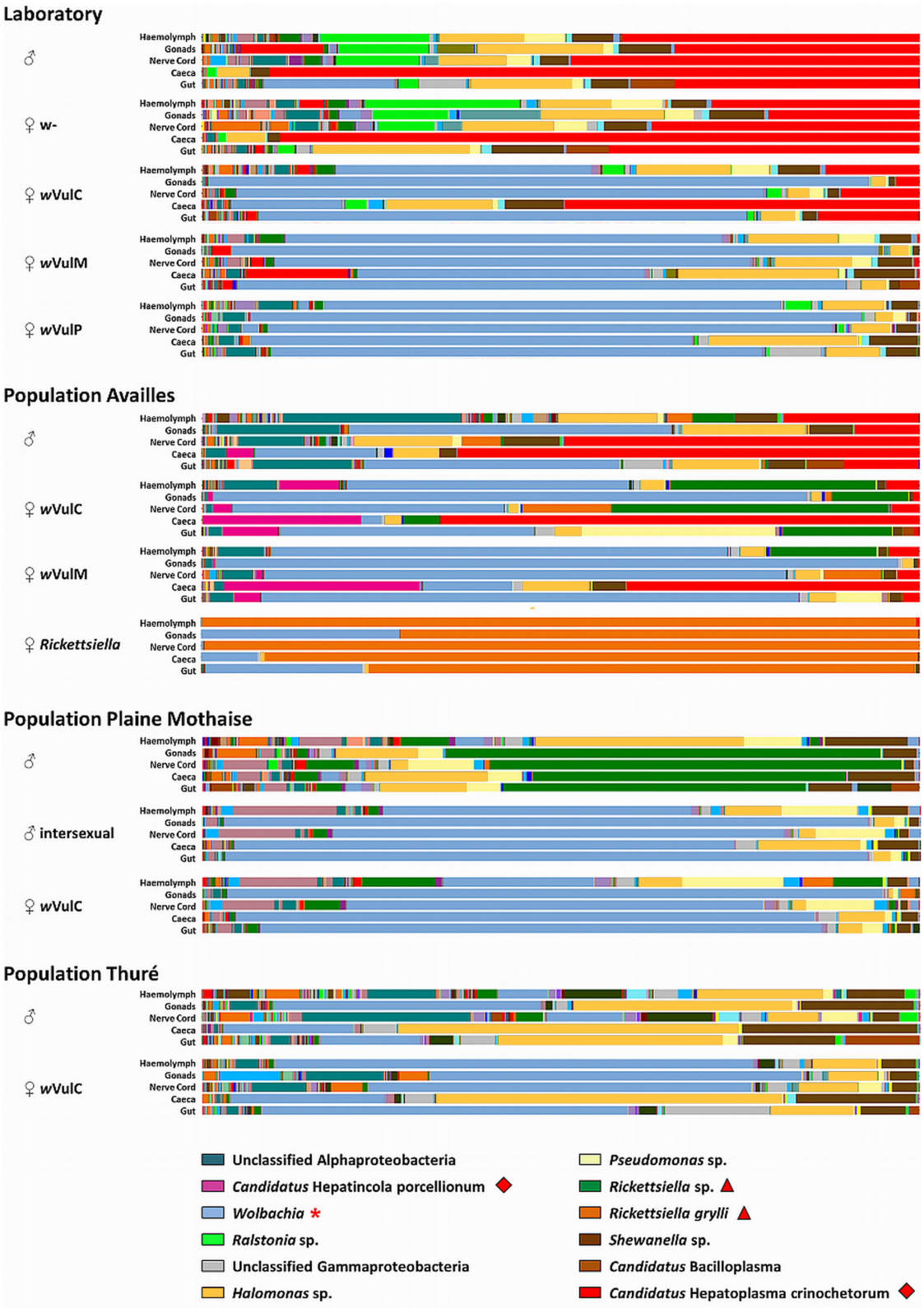

Figure 3. Composition of host-associated bacterial communities (%) in *A. vulgare* from laboratory lineages and three field populations. Each group of five horizontal bars represents the tissue-specific bacterial communities present in a particular host category. Host categories are defined by gender and *Wolbachia* infection status of females. w- indicates absence of *Wolbachia* infection. The legend lists several highly abundant taxa but is not exhaustive. The order of taxa in the legend follows the order of taxa in the community bars (read from left to right). Taxa of particular interest are indicated by red symbols: asterisk = *Wolbachia*; triangles = the bacterial pathogen *Rickettsiella*; diamonds = bacteria previously identified in the caeca and presumably nutritional symbionts.

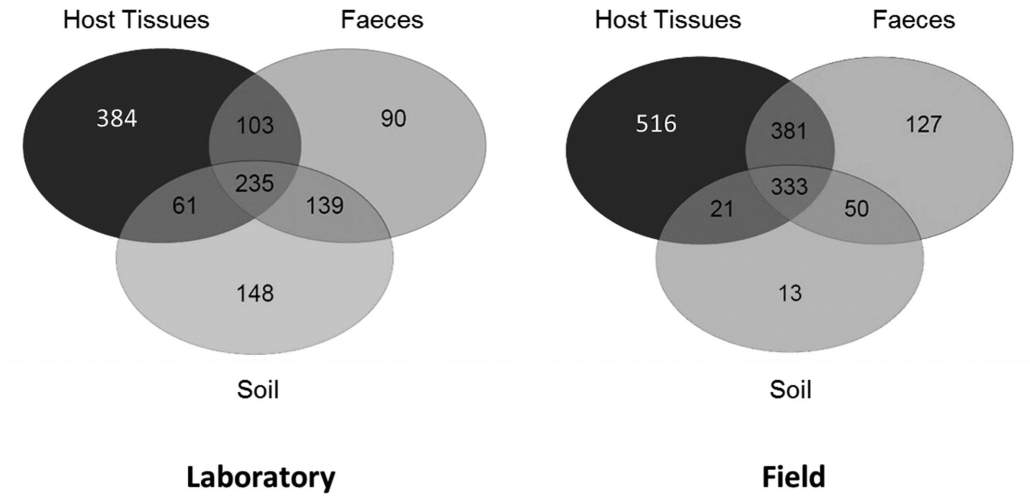

Figure 4. OTU distribution in host-associated, faecal and soil bacterial communities from both laboratory and field samples.

Moreover, the provenance from laboratory or field environments also influenced bacterial community composition, explaining 6.82% of the total variability (Fig. 5a). This impact was visible for both host-associated and faeces and soil communities, indicating that the standardized laboratory environment profoundly alters the bacterial component of *A. vulgare* compared to natural conditions. Taking a closer look at host-associated bacterial communities only, the impact of host origin on microbiota composition became even more apparent, since (i) bacterial communities formed distinct clusters depending on laboratory or field origin of their hosts (Fig. 5a), and (ii) host-associated bacterial communities from different field sites clustered separately when the samples from the laboratory were not included in the analysis (Fig. 5b). This was further supported by an OTU network analysis showing that samples of the same origin (i.e. laboratory and different field populations) shared a higher amount of OTUs than samples of different origins (Fig. 5d).

It has been proposed previously that different host tissues might also represent different microhabitats or niches for bacteria (Goto, Anbutsu and Fukatsu 2006; Dittmer *et al.* 2014). However, the bacterial communities investigated here showed no evidence of tissue-specificity, as illustrated by the absence of tissue-related clusters in both PCoA (Fig. 5c) and OTU network analysis (Fig. 5e). Moreover, two clusters are apparent in Fig. 5c, which correspond to tissues from specimens of laboratory and field origin, respectively, without any tissue-specific clusters within these groups. While the results presented here are based on unweighted UniFrac distances, the same PCoA analyses based on weighted UniFrac distances still do not reveal any tissue-specific patterns (Fig. S2, Supporting Information). Hence, host origin is a more important factor shaping the host-associated microbiota in *A. vulgare* than tissue localization. The same pattern was observed when examining the relationships between bacterial communities at the host individual level using a neighbour-joining tree (Fig. 6). Interestingly, at this scale it appeared that *Wolbachia* infection might also influence bacterial community composition, albeit to a lesser degree (i.e. within the same host population, Fig. 6). While our data do not allow any further conclusions, this aspect deserves more attention since *Wolbachia*-microbiota interactions remain as yet basically unexplored.

The differences between bacterial communities in specimens from different host populations were further substantiated by network analyses of non-random taxon co-occurrence patterns (Fig. 7). First, the number of significant co-occurrence events varied depending on host origin, the highest number being observed in the bacterial communities from laboratory lineages (N = 31), compared to only 9–21 in microbiotas from field populations (Plaine Mothaise: 9; Availles: 10; Thuré: 21), nicely illustrating the higher stability of the laboratory environment. Second, the majority of OTUs involved in these co-occurrence events were assigned to bacterial phyla or taxa that are frequently encountered in diverse terrestrial or aquatic environments (e.g. Actinobacteria, Candidate Division TM7, Flavobacteria, Sphingobacteria, *Acinetobacter* and *Rhizobium*) and/or ubiquitously present in various animal hosts (e.g. *Bacillus*, *Pseudomonas*). Nevertheless, bacterial communities of different origins exhibited distinct co-occurrence patterns. As such, OTUs assigned to the Candidate Division TM7 of environmental bacteria appeared almost exclusively in samples from the Plaine Mothaise, a wetland area that can be expected to represent different environmental conditions compared to the other sampling sites or laboratory conditions (Fig. 7). Taken together, these results further support the hypothesis that the microbiota of *A. vulgare* contains a variable component of environmental bacteria in addition to several highly abundant and more strictly host-associated symbionts, resulting in varying bacterial communities depending on host origin or population.

Predictive functional profiling of bacterial communities

The fact that specimens of different origins harbour different bacterial communities leads to the question whether these symbiotic communities also differ in terms of bacterial functions (as this might have profound effects on the host) or whether they are functionally similar despite the differences in bacterial community composition. To address this question, functional profiles of the different bacterial communities were predicted using PICRUSt (Langille *et al.* 2013) in the absence of shotgun metagenomic data. The accuracy of the predicted metagenomes for tissue samples was considered very high, with NSTI values being $\leq$0.03 for 39 and $\leq$0.05 for 50 out of the 70 host-associated bacterial communities, indicating that the

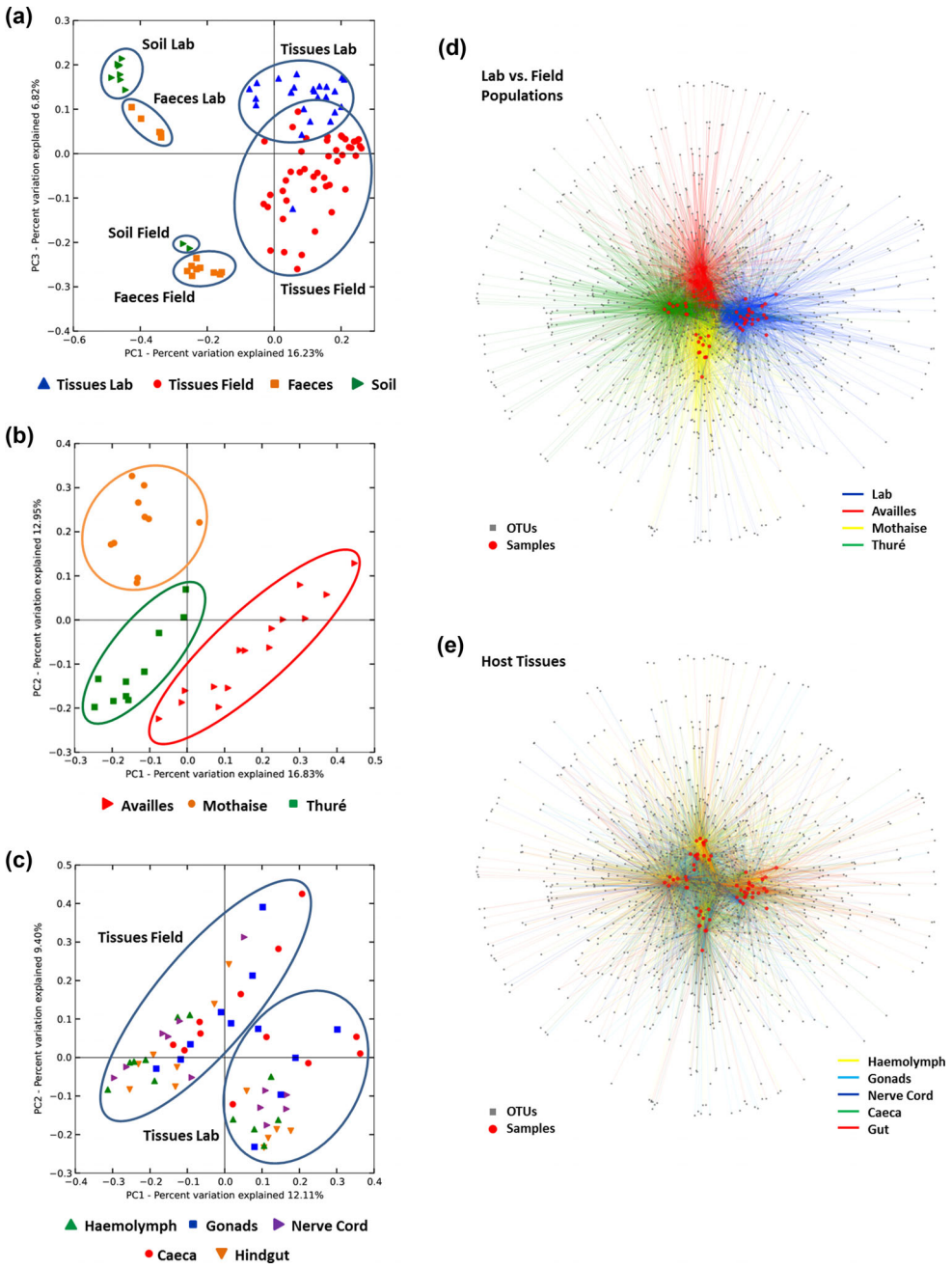

Figure 5. (a–c) PCoA showing differences in bacterial community composition **(a)** between host-associated, faecal and soil bacterial communities, **(b)** between different field populations and **(c)** depending on different host tissues. Each data point represents the microbiota present in a single amplicon pool. **(d–e)** Bacterial community networks connecting OTUs (grey squares) to the samples (red circles) in which they were observed. Samples are clustered depending on the number of shared OTUs. Connectors are coloured depending on host origin **(d)** and host tissues **(e)**. While OTUs are clearly partitioned between hosts from different populations, no distinct pattern is observed depending on host tissues.

closest sequenced genomes used for gene content inference belonged at least to the same bacterial genus. Only eight bacterial communities had relatively high NSTI values (from 0.20 to 0.29) and most of them corresponded to the caeca of hosts from laboratory lineages and the Availles population harbouring the caeca-associated bacteria *Hepatoplasma* and/or *Hepatincola*. Considering that (i) the genome of *Hepatoplasma* has been sequenced only recently (and is therefore not included in the current PICRUSt reference database) and was found to represent a novel lineage within the Mollicutes (Leclercq *et al.* 2014) and that (ii) no genome sequence is available for *Hepatincola*, the higher distance between bacterial communities containing these bacteria at high abundances and their closest sequenced relatives is not surprising.

Interestingly, the different bacterial communities were globally highly similar in terms of bacterial functions, regardless of host origin (Fig. 8a). The latter indicates a certain amount of functional redundancy in the bacterial communities from different host populations, despite different community compositions. However, a few differences became apparent when looking at particular pathways. For instance, the following pathways were more highly represented in bacterial

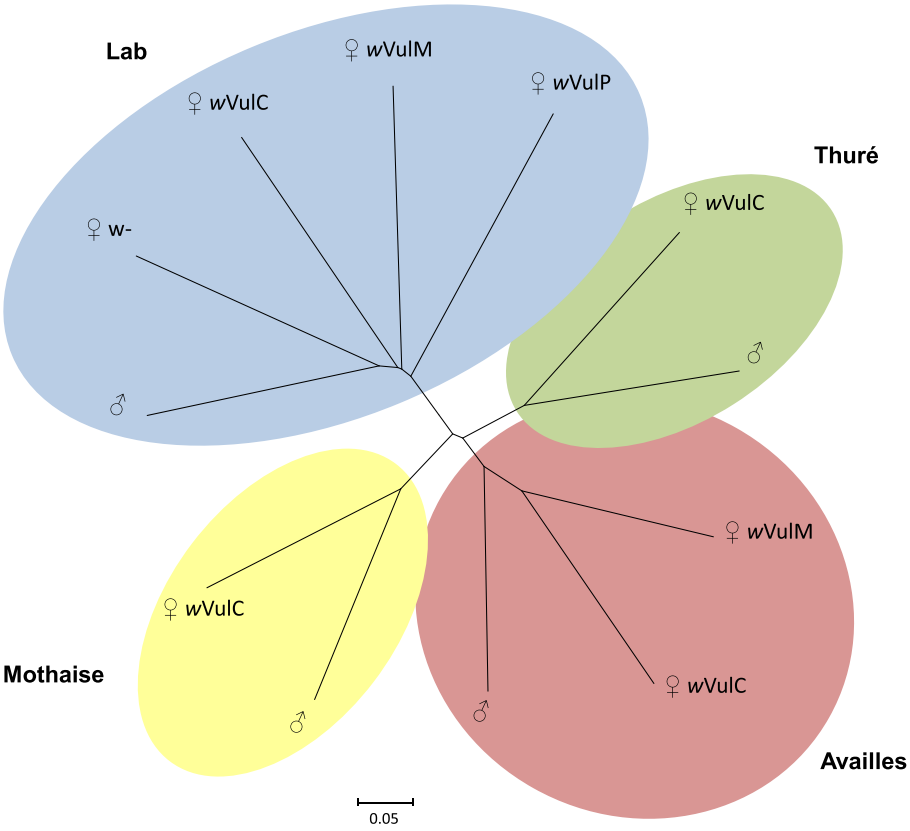

Figure 6. Neighbour-joining tree based on unweighted UniFrac distances showing the phylogenetic relationships between bacterial communities depending on host origin and *Wolbachia* infection status.

communities from field populations compared to those from laboratory lineages: metabolism of cofactors and vitamins (Welch's *t*-test, $P = 0.013$), glycan biosynthesis and metabolism (Welch's *t*-test, $P \leq 0.0001$) as well as transport and catabolism (Welch's *t*-test, $P = 0.0012$). Extending this comparison to the three different field populations revealed that the higher amount of genes involved in the glycan biosynthesis and metabolism pathway was mainly contributed by bacterial communities from Availles (Tukey multiple comparison test, $P < 0.001$); while the transport and catabolism pathway was more highly represented in bacterial communities from Plaine Mothaise and Thuré than in those from the laboratory (Tukey multiple comparison test, $P < 0.001$ and $P < 0.05$, respectively) (Fig. 8b). Several other pathways were differentially represented in microbiotas from different field populations and these differences were most strongly pronounced between bacterial communities from Availles and Thuré (Tukey multiple comparison test, all $P < 0.01$) (Fig. 8b). Pathways that might be of particular interest for future investigations include amino acid metabolism and xenobiotics biodegradation and metabolism. A particular situation was found concerning genes associated with metabolic diseases: Those were more highly represented in microbiotas from Availles than in hosts from other origins (Tukey multiple comparison test, all $P < 0.05$), which might be due to the high abundance of the bacterial pathogen *Rickettsiella* in specimens from this population.

DISCUSSION

This study represents the first large-scale survey of the microbiota associated with the terrestrial isopod crustacean *A. vulgare*. Bacterial communities in faeces and in the surrounding soil were also included, with the aim to assess potential links between the host-associated microbiota and the environmental bacteria it might encounter, for example, via the digestive tract. However, the soil samples sequenced here clearly represent only spot checks and should not be considered representative of the soil bacterioflora. This integrative approach allowed us to identify the major factors shaping microbiota composition in this species. Moreover, it revealed an unexpectedly high bacterial diversity in *A. vulgare*, notably compared to many better-studied insect models whose symbiotic communities are often much less diverse but highly specialized (Koch and Schmid-Hempel 2011; Martinson *et al.* 2011; Wong, Ng and Douglas 2011; Russell *et al.* 2013). The fact that several hundred bacterial taxa can be encountered in a single individual of *A. vulgare* places this species in the same league as termites in terms of bacterial diversity (Hongoh *et al.* 2005; Hongoh 2010). Interestingly, Colman, Toolson and Takacs-Vesbach (2012) demonstrated in a large-scale meta-analysis of gut bacterial communities in diverse insects that bacterial diversity was (i) strongly influenced by host diet and (ii) highest in xylophagous and detritivorous insects such as termites. Hence, the ecological parallels between

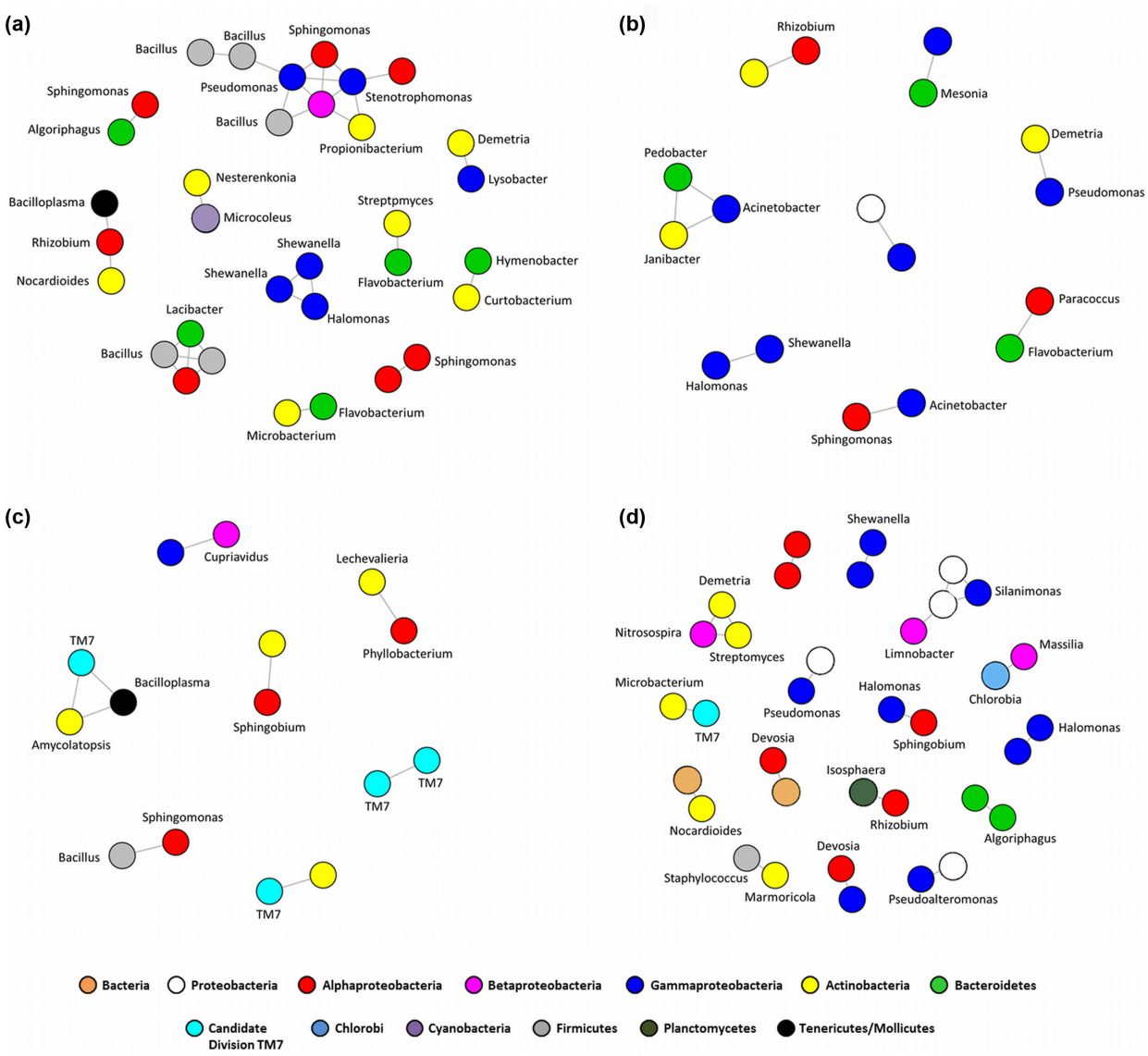

Figure 7. Microbial co-occurrence networks for bacterial communities associated with hosts from (**a**) laboratory lineages, (**b**) Availles, (**c**) Plaine Mothaise and (**d**) Thuré. Nodes are coloured according to phylum/class and annotated at genus level where possible.

terrestrial isopods and termites, which are both decomposers of plant-derived organic matter, might account to some degree for the high bacterial diversity found in both groups of animals. However, taxonomic richness is only one feature of bacterial communities and differences in other aspects, such as bacterial density, might still result in functional differences between termites and terrestrial isopods. Alternatively, such a high diversity might as well be more common in crustaceans than in insects. Unfortunately, microbiota studies in crustaceans are still extremely rare and most of the existing work has been done in aquatic organisms, such as the freshwater flea *Daphnia* sp. (Qi *et al.* 2009), making comparisons with terrestrial isopods difficult. Nonetheless, the microbiota associated with *Daphnia* has been shown to be highly diverse, with 123 bacterial genera detected in *Daphnia pulicaria* (Qi *et al.* 2009). More studies in different crustaceans are needed to confirm whether this is a general trend in this group of arthropods.

One of the major questions addressed in this study was whether different host tissues might constitute heterogeneous microhabitats for bacteria, with certain bacterial taxa being particularly adapted to certain tissue habitats (e.g. the digestive tract), thereby creating tissue-specific bacterial communities. However, bacterial community composition was found to be rather homogeneous between different host tissues, at least in terms of presence or absence of bacterial taxa. An interesting finding in this context is the fact that most tissue-associated microbiotas were predominated by a handful of highly abundant bacterial taxa, indicating the presence of symbiotic bacteria specifically adapted to the host environment. Furthermore, these bacteria represented a large scope of host-symbiont interactions, consisting of a reproductive parasite (*Wolbachia*), bacterial pathogens (*Rickettsiella*) and potential nutritional symbionts (*Hepatoplasma* and *Hepatincola*). It should be noted that all of these symbionts are facultative for the host and therefore not required for host survival. While these taxa had been detected previously in *A. vulgare*, the data presented here provide new insights regarding these symbiotic associations. Hence, we show that *Wolbachia* is not only present in all major host tissues

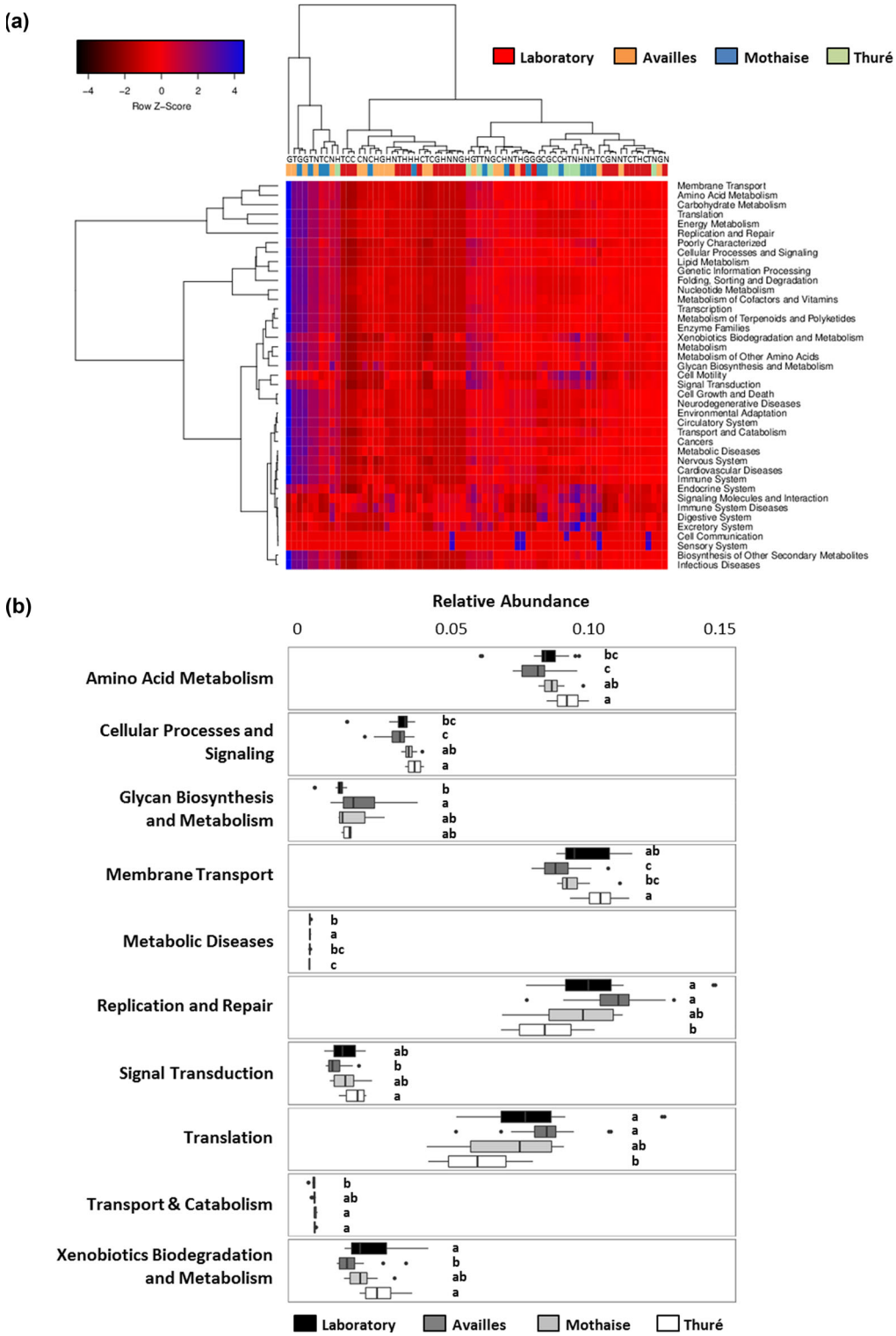

Figure 8. Predictive functional profiles of bacterial communities depending on host origin. (**a**) Heatmap showing predicted gene abundances across all KEGG pathways. No distinct clustering depending on host origin is observed. Letters under the dendrogram indicate host tissues: C = caeca, G = gonads, H = haemolymph, N = nerve cord, T = hindgut. (**b**) KEGG pathways differentially represented in different host populations. Letters indicate significant differences.

as previously reported (Dittmer *et al.* 2014), but also represents the predominant taxon in most, if not all, tissue-associated bacterial communities of infected females. Furthermore, our results illustrate that *Wolbachia*–host interactions can be more complex in natural populations compared to controlled laboratory conditions. This is exemplified by the occurrence of males and females with low *Wolbachia* titers as well as an intersexual male in our field samples. Intersexuality reflects an incomplete feminization, possibly due to a delayed action of *Wolbachia* and/or lower *Wolbachia* titers in the early stages of development (Legrand and Juchault 1969; Legrand, Juchault and Mocquard 1974).

This study is also the first to detect two different strains of the bacterial pathogen *Rickettsiella* naturally infecting the same host species within the same population. Both strains were detected in bacterial communities from various host tissues in specimens from two geographically distant field populations, indicating that infection with this bacterial pathogen is relatively frequent in natural populations.

Two other interesting symbionts of terrestrial isopods are the mollicute *Hepatoplasma* and the Alphaproteobacterium *Hepatincola*. Both bacteria have been previously detected extracellularly in the midgut caeca of numerous terrestrial isopod species including *A. vulgare* (Wang, Brune and Zimmer 2007; Fraune and Zimmer 2008). It has been suggested that bacterial symbionts present in the caeca of terrestrial isopods might play an important role for host nutrition via the provisioning of cellulolytic enzymes (Zimmer *et al.* 2001, 2002a; Zimmer and Bartholmé 2003; Wang, Brune and Zimmer 2007). However, direct evidence for cellulolytic capacities of caeca-colonizing bacteria is still lacking. The data presented here provide new insights into the symbiotic relationship of these bacteria and their terrestrial isopod hosts since we show for the first time that their presence is not restricted to the caeca. Although they were most abundant in the caeca, suggesting that this tissue indeed constitutes their principal niche, both taxa occurred in all tested tissues and *Hepatoplasma* was also detected in the faeces. The latter is of particular interest since both taxa are most likely environmentally transmitted. However, the low representation of this taxon in the faeces renders a systematic transmission via coprophagy rather unlikely. Moreover, it remains to be tested whether *Hepatoplasma* is still viable in the faeces and able to survive long enough to be ingested by a conspecific.

A major finding of this study is the identification of host origin as the most important factor shaping microbiota composition in *A. vulgare*. Hence, bacterial communities not only differed between hosts from laboratory and field environments but also between hosts from different field populations. Moreover, a relatively high number of bacterial taxa occurred in both the host-associated and environmental (i.e. faeces and/or soil) bacterial communities, despite the fact that the microbiotas from host tissues were clearly distinct from bacterial assemblages in faeces and soil, both in terms of bacterial richness and diversity as well as community composition. This suggests that certain members of the host-associated microbiota are specifically adapted to the host environment (such as endosymbionts) while others are probably acquired from the external environment. Consequently, the observed interpopulational variations are likely due to site-specific differences in the environment-related component of the host-associated microbiota, as evidenced by the site-specific bacterial co-occurrence networks dominated by bacteria frequently occurring in terrestrial habitats. This important impact of environmental bacteria might be best interpreted in light of terrestrial isopod feeding habits. In fact, terrestrial isopods constantly ingest bacteria colonizing their food sources and this bacterial component of the plant litter might play a role in terrestrial isopod nutrition, either increasing the nutritive value of low-quality food sources via the provisioning of microbial enzymes or as a direct source of easily digestible nutrients (Kautz, Zimmer and Topp 2002; Ihnen and Zimmer 2008). Local differences in terms of prevailing plant substrates and the associated bacteria might therefore produce the observed differences between host populations. Moreover, terrestrial isopods are gregarious and known to ingest their own or their congeners' faeces (coprophagy), presumably to benefit from enzymes produced by the faeces-colonizing microbes (Gunnarsson and Tunlid 1986; Kautz, Zimmer and Topp 2002). Hence, the ingestion of external bacteria during food uptake and the release of new faeces in the environment might constitute an efficient bacteria shuffling between internal and external environments, while at the same time providing a means to homogenize the bacterial communities within the same host population. Interestingly, the host-associated microbiotas were highly similar in terms of bacterial functions inferred from predictive metagenomes, despite the origin-related differences in community composition, indicating a certain degree of functional redundancy between the different bacterial consortia. Nevertheless, several differences between bacterial communities from particular host populations could be detected in pathways involved in diverse metabolic processes (e.g. metabolism of cofactors and vitamins, amino acid and glycan metabolism), strengthening the hypothesis that bacteria play an important, though as yet not fully elucidated, role in terrestrial isopod nutrition. However, more detailed functional investigations based on actual metagenomes or metatranscriptomes will be needed to disentangle the functional contributions of particular bacterial taxa to terrestrial isopods under varying environmental conditions. Eventually, this might enable us to understand whether shifts in the host-associated microbiota could influence the performance of terrestrial isopods as keystone species in terrestrial ecosystems. This is of particular interest in light of the recent finding that isopods also regulate microbial carbon cycling in soils via a top–down control on microbial soil food webs that remains efficient even under global climate change scenarios (Crowther *et al.* 2015).

DATA ACCESSIBILITY

The 16S rRNA gene sequences obtained in this study have been deposited in the European Nucleotide Archive under the study accession number PRJEB8160.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSEC online.

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Conflict of interest. None declared.

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